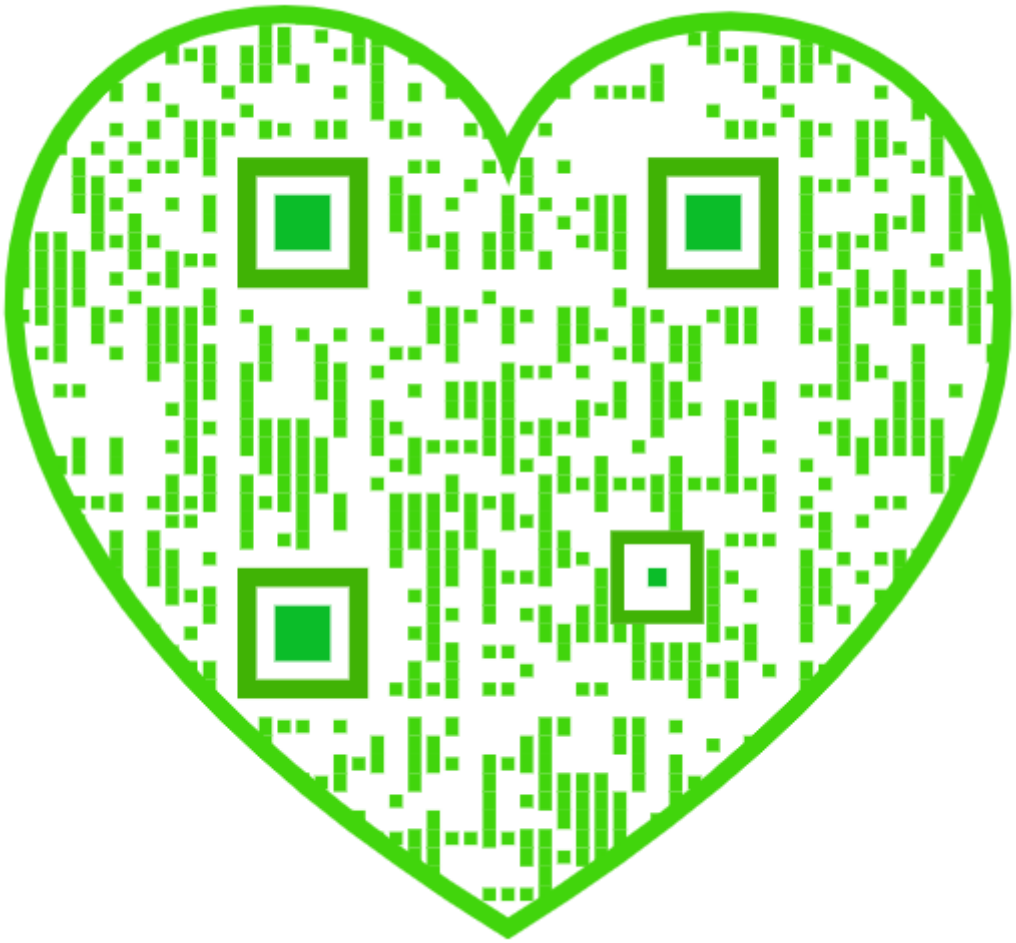

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BIOLOGICAL STAINS

A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory.

By H. J. CONN

New York Agricultural Experiment Station

Chairman, Commission on Standardization of Biological Stains

Prepared with the collaboration of:

J. A. Ambler, S. I. Kornhauser, F. B. Mallory, and L. W. Sharp

Members of the Executive Committee
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PREFACE

WHEN microscopists first began, in the sixties and seventies, to use stains, the demand for dyes for this purpose was naturally too small to justify a special source of supply. They therefore had to make use of textile dyes, which were then very crude and were not constant in their composition. After a number of years, however, the demand for biological stains grew and a special commercial source of supply for them first appeared in Germany. This was the Grübler Co., later Grübler and Holborn. This company did not manufacture the dyes, as used commonly to be thought in other countries; but on the other hand it cannot be denied that its founder made a distinct contribution to science in making the first effort to secure constancy and reliability in dyes intended solely for the use of the microscopist. It is supposed that he tested dyes under the microscope himself, and if a batch proved satisfactory in his experience bought a supply large enough for a number of years, bottled it under his own label and sold it to biologists. There is no question but that in this way the biologist was furnished with a much more reliable line of stains than if he had been obliged to buy directly from the dye manufacturers; but it was an empirical method of standardization and there was nothing to prevent different batches of some dye secured by this company from varying considerably in their composition. Such upon investigation has proved to be the case.

Altho a great service was done to biologists by this company in the latter part of the nineteenth century, such methods of standardization are not in keeping with modern scientific knowledge. A recent cooperative undertaking has therefore been organized in America to put the standardization of stains upon a scientific basis. This undertaking started after the war had caused a shortage of stains, with the object of securing a reliable supply when the foreign sources were unavailable. It has since then been widened in its scope; and now that the foreign products are again available, the purpose of the work is to effect a scientific standardization of stains whether derived from foreign or domestic sources. As a matter of fact, so far only domestic samples have been considered. This has not been because of any prejudice against foreign stains, but because of practical difficulties; it is, in brief, difficult to test each batch before it is put on the market when the concern handling it is in Europe.

The organization thru which this work is being carried on is known as the Commission on Standardization of Biological Stains. It was organized in 1922 under the auspices of the National Research Council and is still affiliated with it, altho now no longer a

part of the larger body. It is in effect a coordinating committee representing the American Chemical Society, the American Society of Bacteriologists, the Society of American Zoologists, the Botanical Society of America, the American Association of Pathologists and Bacteriologists and the American Association of Anatomists. It has a membership of about sixty biologists, members of the various societies just mentioned, who assist in the examination and testing of stains, each in those particular lines of technic with which he is especially familiar. It has secured the cooperation of chemists, dye manufacturers and stain dealers, so as to be sure that the needs of biologists can be immediately reflected in the supply of stains on the market. Its affairs are managed by an executive committee of five members, the present members of which represent bacteriology, botany, dye chemistry, pathology, and zoology, respectively. This executive committee has undertaken the preparation of this book. The authorship of the book has been assumed by the chairman of the committee, however, in order to fix the responsibility and to make bibliographic references to it simpler than in the case of plural authorship; but the assistance of the other committee members in the work has been so great that they may be practically considered co-authors of the book. The chairman of the committee, therefore, wishes to take this occasion to acknowledge the invaluable assistance given by these other members. Without their cooperation such an undertaking would have been impossible.

The chief object of the book is to present in logical form the information which has been accumulating in the hands of the Commission since it was organized. It is neither a treatise on dye chemistry nor one on microscopy; altho it contains information in both fields. It is an effort to present in a form acceptable to biologists the principles of dye chemistry so far as they have a bearing on biological stains; and to discuss the suitability of the different dyes for various biological purposes, presenting data partly original and partly drawn from the literature. The subject matter is realized to be incomplete, particularly that part of it which deals with the biological uses of dyes. An effort has been made to list the most important present uses of stains, and of the obsolete uses to mention those of historical significance; but it is realized that there must be many omissions. It is hoped that readers of the book will cooperate by calling to the author's attention places where the treatment of any subject seems inadequate.

H. J. Conn, *Chairman,*
Commission on Standardization
of Biological Stains.

Geneva, N. Y., 1925.

CHAPTER I

HISTORY OF STAINING

CONSIDERING how dependent microscopists are today upon the use of stains, it is hard to realize that much important work had been done with the microscope before the use of stains was attempted. Altho natural dyes such as carmin and indigo were well known in the early days of the microscope, their use in staining microscopic preparations does not seem to have been mentioned until about 1850; and anilin dyes were not put on the market until 1856. Yet anyone who has studied the history of biology must realize that many discoveries had been made with the microscope before this period.

It is safe to say, nevertheless, that the use of stains revolutionized microscopic technic. The early microscopists were able to make much progress without stains because of their painstaking diligence. The work without stains must have been extremely difficult, and it is hard on reading some of the old publications to believe that some of the minute structures described were actually seen. Few users of the microscope today would be likely to have either the patience or the eyesight to do the work described in those early days. The fact that the microscope is now being used successfully in the hands of so many students who would not think of comparing themselves with the pioneers in microscopy is due to the use of stains more than to any other factor—altho of course no one can deny that modern improvements in the microscope have also played a part of great importance.

The first use of a dye in microscopic work seems to have been by Ehrenberg (1838)*, who did not intentionally stain his specimens, but devised the scheme of grinding indigo and carmin into a very fine powder and feeding them to the microorganisms he was studying. His idea in doing this was that the organisms would consume the dye bodily and that their digestive system could be traced by observing what portions of the body became colored. Ehrenberg found by this method that the organisms in question showed certain zones or bands of color; and assuming that each of these colored spots was a stomach, he named the group of organisms "Polygastrica." Inasmuch as the group included bacteria and protozoa, the name was somewhat of a misnomer. It is interesting, however, to realize that Ehrenberg's technic is still used to demonstrate the ability of protozoa to engulf food particles.

The early history of staining from this time on is given in a very interesting manner by Gierke (1884–5) and by Mann (1902), one

*For references cited see Bibliography pp. 138 to 145.

or the other of which sources is recommended to anyone desiring a more detailed account than is given here.

Besides the fairly common application of iodine to microscopic preparations there seems to have been no further effort to color objects under the microscope until the middle of the century. At this time carmin was used almost simultaneously by two botanists, Cöppert and Cohn (1849) and by a zoologist Corti (1851). Göppert and Cohn used the dye to assist them in studying the rotation of the cell contents of *Nitella flexilis*. Corti's work is overlooked by both Gierke and by Mann; but he states definitely (p. 143) that to observe distinctly the epithelial cells one should color them lightly with a solution of sugar or of carmin in a mixture of half water and half alcohol. This work was very promptly followed by another use of the same dye—the staining of chlorophyl granules in plants —by Hartig (1854 and 1858). In studying the nucleus of cells he made use not only of carmin but of litmus and black ink; and he observed that while albumin and gelatin were easily stained, the dyes had no action on such material as gums and mucin. Altho these authors did careful scientific work and certainly were the first users of dyes for histological purposes, their work apparently attracted no attention at the time. The real introduction of biological stains was made by Gerlach (1858).

Altho Gerlach did not discover the action of dyes on microscopic objects, nor was he the first to use carmin, nevertheless he should be, and generally is, considered the father of the technic of staining. Having observed that tissues became colored after injection with a poorly prepared carmin gelatin, he devised the scheme of preparing an ammoniacal carmin. He was therefore the first to use ammonium carminate, which is so nearly indispensible to modern histologists. His early efforts with it, however, were unsuccessful—until he had a lucky accident which revealed the source of his trouble and opened up the way for further work. He happened to leave a section of nervous tissue, which had been hardened in potassium bichromate, over night in a very dilute carmin solution. When he examined it twenty-four hours later he found that it was beautifully stained, with fine differentiation of nerve fibers and nerve cells. His earlier failures had been due to the use of too strong a solution of the dye. This gave the key which helped solve the problem of tissue staining. Advances came quickly after that; for Gerlach had shown the way, and others merely had to follow.

These advances were not wholly with carmin; altho at first the anilin dyes were scarcely known and the number of stains available to microscopists was quite limited. Indigo was first employed by Maschke (1859) who was familiar with Hartig's work but not with Gerlach's. It is stated (anonymous, 1865) that Thiersch and Müller had just developed a technic employing carminates in combination with oxalic acid, and a year later Schweigger-Seidel and

Dogiel (1866) introduced a combination of carminates with acetic acid. Haematoxylin was first introduced as an histological stain by Böhmer (1865), altho a previous rather unscientific attempt had been made by Waldeyer (1864) to stain axis-cylinders by means of the watery extract of logwood. Böhmer's greater success was due to his use of haematoxylin crystals in combination with alum, either by accident, or else because he knew that it was frequently used as a mordant in textile dyeing. Slightly later Frey (1868) showed that similar results could be obtained by mixing the mordant with the solution in which the tissues were fixed before they were stained.

Double staining was introduced at about this same period, when Schwarz (1867) proposed fixing tissue in creosote and acetic acid, then staining 24 hours in very dilute ammonium carminate, and subsequently washing and staining for two hours in picric acid. A year later Ranvier (1868) first used a picro-carmin stain to obtain the same results by a single procedure.

Anilin dyes had become commercial articles before all these advances with the natural dyes had been made, the first one having been introduced in 1856 when Perkin prepared mauveine. Fuchsin, under the name of anilin red, appeared in 1858. The first suggestion of their use in histology seems to have been made by Beneke (1862), who used acetic acid colored with a lilac anilin, probably a mauveine or anilin violet; while two years later Waldeyer (1864) used anilin red (fuchsin) and also a blue and a violet anilin dye. The latter investigator observed the ability of fuchsin to stain nuclei more deeply than cytoplasm and the axis cylinder more deeply than the medulary sheath of nerves.

The principle of differentiation following staining was soon introduced. Böttcher (1869) differentiated his sections by partially decolorizing with alcohol after staining with rosanilin nitrate. A very similar method was later published by Hermann (1875), who is often mistakenly given the credit for originating the principle. Later the same procedure was further investigated by Flemming (1881) who tried both acid and basic dyes, finding that the method was satisfactory only in the case of the latter group.

Gierke (l. c.) in his historical discussion of staining says that the history up to his day (1884) was divided into three periods, each occupying a decade. The first decade, the fifties, was characterized by a few important but unrelated discoveries, which ended in the work of Gerlach, each investigator following up accidental observations on the staining powers of carmin and the other well-known dyes of those days. After Gerlach's work the development of the technic in the sixties was more rapid and depended less upon chance success by the individual investigator; the effort was made to use similarly all the dyes and metallic colors then available. The next decade would have had much less left to develop in this line if it had not been that by this time the great variety of anilin dyes

were available and microscopists were constantly finding new uses for them. Gierke wondered if there would be any opportunity for equal development during the ten years to follow his paper.

That development did not stop in his day is well known. Scarcely a year has passed without the introduction of some new staining technic of considerable importance. Sometimes dyes hitherto unknown to the biologist have been shown to be valuable in bringing out some particular structure; at other times new combinations of dyes have proved of special value for other purposes; while by other investigators it has been shown that old methods, used with modern refinements of apparatus and technic, may bring out details not dreamed of by the early histologists. But the farther this work has progressed the more the microscopist has become dependent upon his supply houses to furnish him reliable stains, so carefully purchased or manufactured that each lot ordered could be counted upon to duplicate the last.

The preface of this book describes briefly how a company was formed in Germany to meet the demand for dyes for staining purposes that developed during the last three decades of the nineteenth century, and how the recent post-war conditions, together with the modern demand for a more scientific basis for the industry, led to the establishment in America of a Commission on Standardization of Biological Stains. The work of the Commission is two-fold. First, by cooperation of various biologists and chemists it is planning to get together all the available information concerning the nature of dyes as related to their use for various purposes in microscopic technic; secondly, by working with the manufacturers it is trying to see that the supply of stains available in America is of the highest possible quality, as judged by their performance in actual laboratory use.

The first of these purposes has been partly accomplished by a series of brief notes appearing in certain biological publications; and is now being more fully realized by the publication of this book. The second object is being slowly brought about by the plan of certifying stains. Manufacturers of stains are being encouraged to submit to the Commission, for testing, samples of different batches of their various stains. These samples the Commission compares by chemical tests and by submitting them to several biologists skilled in different lines of miscoscopic technic. Then in the case of those samples which prove satisfactory, the Commission allows the company to sell the batch tested with a label on it issued by the Commission, bearing the statement: "Found satisfactory by Commission on Standardization of Biological Stains for purposes mentioned on main label. Use for other purposes not contraindicated unless specifically so stated on said label." This certification is issued only for the particular batch tested. The manufacturer is furnished only with enough labels to last for that batch in the ordinary course of trade; and occasional tests of market

samples are made to see if any samples sold under the label differ from the one originally tested. In this way it is hoped before long to have all the stains on the American market from batches which have passed chemical tests and have also been tested and approved by biologists skilled in their use.

When stains are put on the certification basis, specifications for them, based on the Commission's tests, are prepared and future lots of stain submitted for certification are in all cases expected to fulfill these specifications. So far as these specifications have already been drawn up they are listed in the appendix of this book.

CHAPTER II

THE GENERAL NATURE OF DYES AND THEIR CLASSIFICATION

DYES are generally classed in two groups, the natural and the artificial. The former class is now of relatively smaller importance from the standpoint of the manufacturer and the textile dyer; for the artificial dyes far outnumber them and the advancement of science is gradually making it possible to produce many of the formerly natural dyes by artificial means. It just happens that one or two natural dyes, the derivatives of cochineal and logwood extract (see Chap. IX) are among the most valuable biological stains; but the natural dyes in general are so few in number that they can be practically disregarded in considering the general chemical nature of dyes.

Because the first artificial dyes were produced from anilin, all of this class are often called "anilin dyes," altho there are now a large number of them which bear no relation to this compound and are not derived from it. Therefore the term is now quite largely being replaced by the more correct expression "coal-tar dyes," since all of them are made by chemical transformations from one or more substances found in coal-tar.

BENZENE

All coal-tar dyes may be considered as derivatives of the hydrocarbon, benzene, C_6H_6, which is the mother substance of the very important aromatic series of organic compounds. It is an unusual chemical compound in many respects, and it will be well, in order to understand the structure of dyestuffs, to review briefly one theory of its structure which accounts for many of its properties. The molecule of benzene is composed of six carbon atoms combined with six hydrogen atoms in such a way that each hydrogen atom is identical in all its reactions with every other hydrogen atom in the molecule. Now a carbon atom is considered to have in all cases four valency bonds, that is it is capable of uniting chemically with

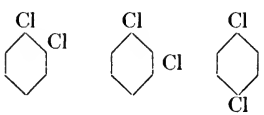
four atoms of hydrogen which has a valency of one. The simplest and best way of expressing these facts by a structural formula is shown in the figure:

The double bonds in this ring cannot be considered as stationary; for, if they were, a compound in which two adjacent hydrogen atoms had been replaced by other elements or radicals should occur in two different forms according to whether there were a single or a double bond between the two carbon atoms to which the substituting elements or groups were attached—which never proves to be the case. Hence the double bonds must be considered as mobile, each pair continually oscillating back and forth between the carbon atom bearing it and the two adjoining carbon atoms.

In practice the formula for benzene is abbreviated to a simple hexagon:

in which each corner represents a carbon atom. If no chemical symbol is placed outside the ring at any corner, it is understood that an atom of hydrogen is attached at that point. This configuration is spoken of as the "benzene ring." When the symbol of some element or radical is written at a corner, it means that the hydrogen atom at that point has been replaced by the element or radical to which the symbol refers.

When two hydrogen atoms are replaced there are only three possible positions in the molecule which the replacing groups, or substituents, can take, as shown by the following figures, using chlorine as the substituent:



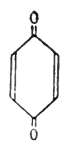
In the first formula, the substituents are said to be in the "ortho" position to each other; in the second they are in the "meta" position, and in the third in the "para" position. These three compounds are called respectively: ortho-dichlorobenzene, meta-dichlorobenzene, and para-dichlorobenzene. The three prefixes are commonly shortened to the respective initials: "o-" "m-," and

"p-." When the compound is complex it is customary to number the corners of the ring thus:

In naming a compound in this way, the number of the corner to which a group is attached is given immediately before the name of the group. Thus, the three compounds shown above may be called respectively: 1, 2-dichlorobenzene, 1, 3-dichlorobenzene and 1, 4-dichlorobenzene.

There is another type of substitution in the benzene ring which is very important in dye chemistry. Two atoms or groups having two valency bonds instead of one may also replace two hydrogen atoms, provided the replacement takes place simultaneously and the hydrogen atoms replaced are situated either in the ortho or in the para position to each other. Thus two oxygen atoms (which are bivalent) may replace two hydrogen atoms (which are monovalent) forming the compound known as quinone $C_6H_4O_2$, the formula for which is

or as commonly written



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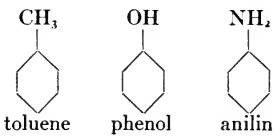
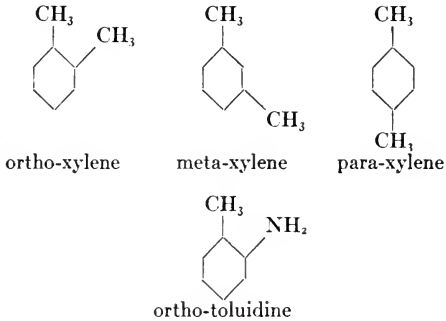
In printed formulae, such as those that follow in this book, the quinone ring is often abreviated still further by omitting the double bonds within the ring. The substituent atoms or groups may or may not be alike, so long as both have two valency bonds entering into the combination. This type of substitution involves a rearranging of the double valency bonds in the benzene ring; and in compounds of this type, called quinoid compounds, the double bonds are supposed to be fixed, not mobile as in benzene. This change of the valency bonds takes place very readily in many dyes, and certain peculiarities of their behavior are explained by it; (see for example p. 84).

Three mono-substitution products of benzene are of importance in considering the structure of dyes, namely; toluene or methyl-

benzene, $C_6H_5 \cdot CH_3$; phenol, carbolic acid or phenylic acid, $C_6H_5 \cdot OH$; and anilin or phenyl amine, $C_6H_5 \cdot NH_2$. Their constitutional formulae are as follows:



Two important di-substitution products are xylene or dimethyl benzene $C_6H_4 \cdot (CH_3)_2$, and toluidine, $C_6H_4 \cdot CH_3 \cdot NH_2$. Both of these occur in the above mentioned three isomeric forms, as shown below for xylene:

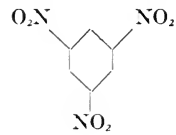


CHROMOPHORES, CHROMOGENS, AND AUXOCHROMES

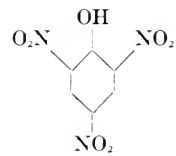
Certain groups of elements are known as chromophores because when they occur in a benzene derivative they impart to the compound the property of color. The benzene compounds containing chromophore radicals are known as chromogens. A chromogen, however, altho it is colored, is not a dye, in that it possesses no affinity for fibers or tissues. It may coat them, but only mechanically, and it will be easily removed by mechanical processes. That is, it will not "take." (See, however, the discussion of fat stains, p. 33). In order for a substance to be a dye, it must contain in addition to the chromophore group, a group which imparts to the compound the property of electrolytic dissociation. Such auxiliary groups are known as auxochromes. They may slightly alter the shade of the dye, but are not the cause of the color. Their function is to furnish salt-forming properties to the compound. Certain chromophoric groups have also slight auxochromatic properties.

To illustrate these different types of groups, let us take a typical example. The nitro group ($-NO_2$) is a chromophore. When three

of these groups displace three hydrogen atoms in a benzene molecule, we have the compound trinitrobenzene,



which is yellow. It is not a dye, however, but is a chromogen. It is insoluble in water, and is neither an acid nor a base; that is, it does not dissociate electrolytically and consequently cannot form salts with either alkalies or acids. If, however, one more hydrogen atom is replaced, this time with the hydroxyl group (-OH), which is an auxochrome, the resulting compound,

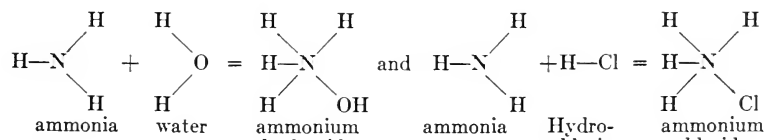


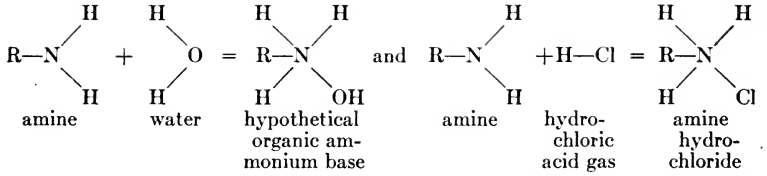
is an acid, capable of electrolytic dissociation and of forming salts with alkalies. It is the familiar substance picric acid, and is a yellow dye.

It will thus be seen that the color of picric acid is due to the chromophoric nitro groups, and that its dyeing properties are due to the auxochromic hydroxyl group. If the nitro groups be reduced to amino groups (-NH_2), which are not chromophores, the resulting compound is colorless and hence is not a dye.

Summing up, we arrive at the definition of a dye as an organic compound which contains chromophoric and auxochromic groups attached to benzene rings, the color being attributable to the chromophores and the dyeing property to the salt-forming auxochromes.

Some auxochromes are basic, e.g., the amino group (-NH_2), while others are acidic, e.g., the hydroxyl group (-OH). The amino group owes its basic character (which it transmits to the whole molecule) to the ability of its nitrogen atom to become pentavalent by the addition of the elements of water (or of an acid), just as in the case of ammonia; thus:





The hydroxyl group, on the other hand, is weakly acidic, as it can furnish hydrogen ions by electrolytic dissociation. The more of either one of these two groups in a compound, the stronger base or acid it becomes. If there is one of each, the basic character of the amino group predominates, but is weakened by the influence of the acidic hydroxyl group. The strength of both groups is also influenced by other groups or atoms in the compound; thus, for example, the chromophore $-NO_2$, altho incapable in itself of conferring acid properties to the compound, exerts an influence to make any hydroxyl group in the compound more strongly acidic, in other words to become more highly dissociated electrolytically.

One other group of atoms encountered in dye chemistry needs explanation, namely the sulfonic group, $-SO_3H$. It is a salt-forming group of strongly acidic character, in that it suffers extensive electrolytic dissociation. This group, however, is only very feebly auxochromic. Its function is to render a dye soluble in water, or to change an otherwise basic dye into an acidic one, as in the case of the fuchsins, where the strongly basic "fuchsins" are changed into the strongly acid "acid fuchsins" merely by the introduction of sulfonic groups into the former. A compound which contains a chromophore group and a sulfonic group is not a dye, however, unless there is also present a true auxochrome group.

From what has been said above, it is not to be presumed that the dyes of commerce are actually bases or acids. Generally the basic dyes are sold as salts of a colorless acid, such as hydrochloric, sulfuric, oxalic or acetic acid. Likewise the acid dyes are sold as their sodium, potassium, calcium or ammonium salts. Occasionally the basic dyes are sold as the free bases, as for example the oil soluble dyes (see p. 38). When a basic dye which is ordinarily sold in the form of a salt comes into commerce as the free base, it is customary to use the word "base" immediately after the name of the dye. Thus, "basic fuschin" indicates a salt of fuchsin with a colorless acid, while "fuchsin, base" indicates fuchsin itself, not combined with an acid.

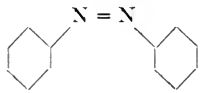
THE CHROMOPHORES

As stated above, every dye contains at least one group of atoms known as a chromophore, which is regarded as being responsible for the colored properties of the compounds in which it occurs. Some of these chromophores have a basic character, others acid. There are only a comparatively small number of them which enter

into the usual biological stains, and only these need be considered here. They are as follows:

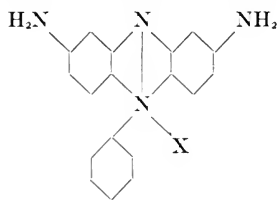
BASIC CHROMOPHORES

1. *The azo group*, —N=N—, which is found in all azo dyes, of which methyl orange and Bismarck brown are well known examples. In all these dyes, a benzene ring is attached to each nitrogen atom. All the dyes of this group may be looked upon as derivatives of azobenzene,

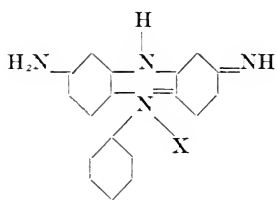


2. *The azin group*,

which is found in phenazins, of which neutral red and the safranins are good representatives. The skeleton formula of a safranin is:

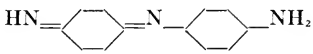


in which x represents the negative ion of a monobasic acid such as hydrochloric, acetic, nitric or sulfuric. This chromophore is capable of variety of rearrangements of its valency bonds, as the bond between the two nitrogen atoms may disappear and the compound assume a quinoid structure, as for example the following grouping:

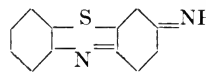


3. *The indamin group*, —N=, as observed in the indamins, thiazins, and so forth. Methylene blue is the best known representative of this group. In these dyes, two benzene rings are at-

tached to the nitrogen atom, one of these being in the quinoid form and hence adding a second chromophore. The typical indamin formula is:

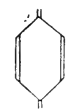


In the thiazins, such as methylene blue, the two benzene rings are further joined together by a sulfur atom, forming three closed rings of atoms. The simplest thiazin base would be:

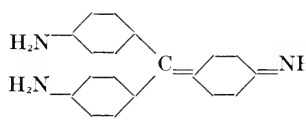


ACID CHROMOPHORES

1. *The nitro group*, $-NO_2$, as in picric acid.
2. *The quinoid benzene ring*,



which occurs in a long series of dyes, such as the indamins above mentioned, the xanthenes and the di- and tri-phenyl methanes, which include many well known stains, such as rosolic acid, fuchsin, methyl green and the methyl violets. A typical triphenyl methane formula is that of pararosanilin, base:

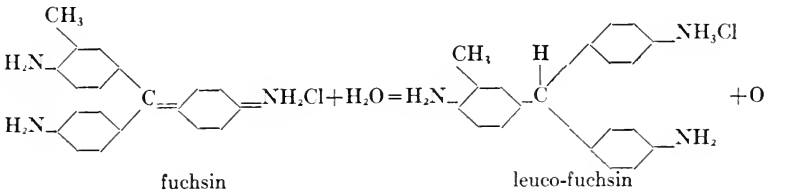


LEUCO COMPOUNDS

The different chromophores differ considerably from one another, but they all have one property in common. In the language of chemistry, they all have unsatisfied affinities for hydrogen; or in other words, they are all easily reduceable, for combining with hydrogen is the opposite of oxidation and is, therefore, reduction. The nitro group may be reduced to an amino group; in the azin group the bond between the nitrogen atoms may break and two hydrogen atoms be taken on; while in the various chromophores with double bonds (such as the quinoid ring) the double bond may break and hydrogen atoms become attached to the valences thus freed.

Now in every case this reduction destroys the chromophore group, and as a result the compound loses its color. In other words a dye retains its color only as long as its affinities for hydrogen are

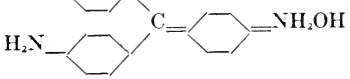
not completely satisfied. These colorless compounds are known as leuco compounds; thus fuchsin yields leuco-fuchsin on reduction, and methylene blue reduces to leuco-methylene-blue. For example:



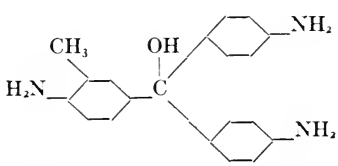
fuchsin leuco-fuchsin

Ordinarily this reaction is reversable under conditions favoring oxidation. It is of especial significance to the bacteriologist, as dyes can often be used as indicators of reduction.

Certain dyes form a still different type of leuco compound, often called a "leuco-base." We have seen that the basic dyes ordinarily occur as salts of some colorless acid; now, in the case of certain dyes, notably the tri-phenyl methanes and xanthenes (Chapters VI and VII), as soon as the acid radical is removed, the compound becomes colorless. This is because a rearrangement of the atoms in the molecule takes place upon neutralization so as to give, not the true dye base, but a compound known as a carbinol (see p. 59) in which the chromophore does not occur. Thus the theoretical base of fuchsin which should be obtained upon removal of the acid radical is:



The compound actually formed, however, is the pseudo-base or carbinol:



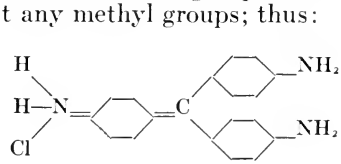
In this compound, it will be readily seen, there is no chromophore; hence it is colorless. These pseudo-bases are of little significance to the biologist, but they are of importance to the dye manufacturer as intermediates in the preparation of dyes.

In the case of many acid dyes the chromophore is similarly broken by a rearrangement of the atoms which occurs on neutrali-

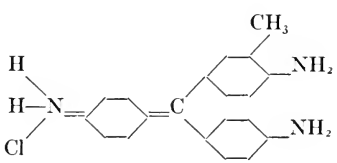
zation. This reaction is ordinarily very readily reversable and makes such dyes useful indicators of acidity. It is discussed more fully under acid fuchsin (p. 64) and phenolphthalein (p. 83).

CLASSIFICATION OF DYES

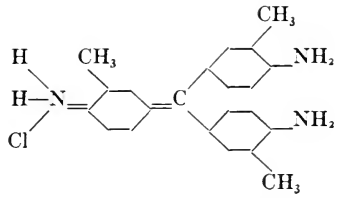
On the basis of the chromophore present the simple synthetic dyes are classified into several groups. If each of these groups were characterized by a single color or by a few closely related colors, dye chemistry would be a comparatively simple proposition. As a matter of fact a single chromophore may occur in dyes of practically all colors of the rainbow. It is ordinarily impossible to determine, *a priori*, from the chemical formula of a dye what particular color the compound may have; but there is, nevertheless, a certain general rule which correlates chemical formula with color. In any group of compounds, the simpler ones are converted into the more complex by substitution of radicals for hydrogen atoms. In the dyes the substituents are generally methyl or ethyl groups, or sometimes phenyl groups. Now the general rule is that the larger the number of hydrogen atoms that have been replaced by these groups the deeper the color. The tendency is for the color of the simplest dyes in any group of homologous compounds to be yellow, passing thru red to violets and then greens and blues, as the homologs become higher thru the introduction of successively larger numbers of methyl or other substituting groups. Thus the compound pararosanilin, which is very frequently sold as basic fuchsin, but should more properly be called basic rubin, is a triphenyl methane, with an amino group attached to each benzene ring, but without any methyl groups; thus:



Rosanilin, which is similar in composition, but contains one methyl group attached to one of the benzene rings,

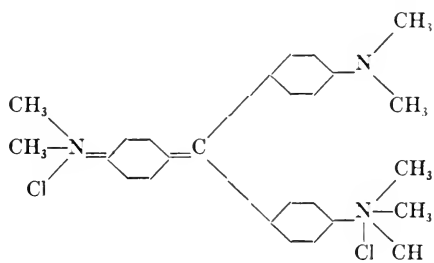


is a red very similar to pararosanilin but with less of a yellowish cast. Now another methyl group may be introduced into each of the other two benzene rings, and each one successively deepens the shade of red, so that the highest homolog of the series, new fuchsin:



has a more bluish cast than any of the others. Thus basic fuchsins can vary considerably in their shade according to the proportions in which these four possible components may be mixed.

It is also possible in another way to deepen the color of pararosanilin still further, namely by introducing methyl groups into the amino radicals instead of directly on the benzene rings. Thus the methyl violets are obtained; and the more methyl groups introduced the bluer the violet, until when all six available hydrogen atoms are thus substituted, crystal violet, the deepest of them all, is obtained. By using three ethyl groups instead of methyl, Hoffman violet or dahlia is formed, which is deeper in color than the trimethyl compound, due to the heavier groups introduced. If three phenyl groups (i.e., the benzene ring (C_6H_5-) are introduced instead of methyl or ethyl, the color is still further deepened, the resulting dye being spirit blue. Further, it is possible to introduce another methyl group into crystal violet, by addition of methyl iodide (or chloride) to one of the trivalent nitrogen atoms, whereby its valency is increased to five, and a green dye, methyl green, is produced:



With these facts in mind it will be seen that the grouping of dyes as based upon these chromophores does not classify them in relation to their color. It is a useful classification, however, because it puts together those that have similar chemical structure. The important biological dyes, thus classified, fall into the following groups:

1. The nitro dyes.
   e.g., *picric acid.*
2. The azo group.
   e.g., *methyl orange, Bismark brown, orange G, congo red, Sudan III and Sudan IV.*

3. The oxyquinone group.
   e.g., *alizarin*
4. The quinone-imide group, including
   (a) Indamins
   (b) Thiazins; e.g., *thionin*, *toluidine blue*, *methylene blue.*
   (c) Oxazins; e.g., *brilliant cresyl blue*, *Nile blue.*
   (d) Azins, including
      (i) Amido-azins; e.g., *neutral red.*
      (ii) Safranins; e.g., *safranin O*, *madgala red.*
      (iii) Indulins; e.g., *nigrosin.*
5. The phenyl-methane dyes, including
   (a) diphenyl-methanes, e.g., *auramin.*
   (b) Diamino tri-phenyl methanes; e.g., *malachite green*, *brilliant green*, *light green.*
   (c) Triamino tri-phenyl methanes; e.g., *basic fuchsin*, *acid fuchsin*, *methyl violet*, *gentian violet*, *methyl green*, *anilin blue.*
   (d) Hydroxy tri-phenyl methanes (Rosolic acids); e.g., *aurin*, *corallin red.*
6. The xanthene dyes, including
   (a) Pyronins; e.g., *pyronin G* and *B.*
   (b) Rhodamines; e.g., *Rhodamine B.*
   (c) Fluorane derivatives; e.g., *eosins*, *erythrosin*, *rose bengal.*
   (d) Phenolphthalein and the sulphonphthaleins.

DYE NOMENCLATURE

Very little system has been used in naming dyes, and as a result their nomenclature is extremely confused. Generally the manufacturer of a dye which he thinks is new or which he wishes the public to consider a new dye sells it under a new name which is not intended to give any clue as to the nature of the dye. If the manufacturer knows that the name is a mere synonym of one already in use he does not say so, for he wishes to encourage the sale of his own product rather than that of some other dye maker. Accordingly it has been left for others, who are not financially interested, to work out the synonymy of the dyes; and the list of names that are found to apply to a single dye is sometimes amazing.

With the dyes in general so unsystematically named, it is natural that the same confusion should reign in the nomenclature of biological stains. This confusion is very unfortunate, for it often misleads the biologist as to just what he is doing. For example, some histologist may have on hand a bottle of stain labeled dahlia and he may find it useful for some new technic, which he publishes; while another may propose for an entirely different technic the stain Hoffman violet. Then a third laboratory worker may read both articles and wish to try both methods; so he accordingly

orders both dahlia and Hoffman violet. His dealer, who is probably quite unacquainted with dyes, will very likely send him a bottle bearing each name, and the purchaser has no easy way of discovering that the two are identical; so he may continue for years to use the two stains for different purposes, misled by their labels and thinking them distinct. The manufacturers and dealers in stains have sometimes encouraged this confusion by their practice of taking care to have the label on the bottle agree with the name used in the customer's order, regardless as to what the usual name for the dye may be.

An attempt to relieve this confusion has been made by the Commission on Standardization of Biological Stains (1923f) by publishing a list of biological stains with their best known synonyms. In each case one of the names is listed as a preferred designation. Sometimes general usage made it easy to select one name as the preferred one; but in other instances the selection was more or less arbitrary. This same list, with a few revisions in the way of additions and corrections, is given in the appendix of this book (p. 106). The preferred designations in this list are the same as in the earlier, except in the one case of methylene azure. For this stain Azure I was preferred in the earlier list; but as it was merely a trade name of somewhat uncertain application methylene azure seems preferable. The list of synonyms has been revised more extensively, largely to omit names that are obsolete and have no present meaning.

DYE INDEXES

Inasmuch as the dye industry originated in Germany and until the war was almost a monopoly of that country, it is natural that the first serious efforts to index the dyes should have been undertaken in that country. Until recently the only important index of dyes was Schultz's Farbstofftabellen, which is now in its sixth edition (1923). This index lists all of the important textile dyes, giving their synonymy, their chemical composition, methods of preparation, and distinctive characteristics. As these descriptions are concise, it seemed well to refer to the Schultz number of all the stains listed in the article on stain nomenclature above mentioned (Commission, 1923f), wherever such could be given.

More recently another dye index has been published in England by the Society of Dyers and Colourists (1923). This publication, known as the Colour Index, is more complete than even the sixth edition of Schultz, and lists even such dyes as narcein, thionin and iodine green, which are no longer of use in the textile industry and have been omitted from recent editions of Schultz. The synonymy is more complete and up-to-date than that in Schultz, and many more chemical formulae are given. Accordingly in the following pages the stains are denoted by their Colour Index number (abbreviated C. I. No.) instead of by their Schultz number, as in the

list previously published. The Schultz number of each of them is given for reference purposes, however, in the list in the appendix, p. 106.

DYE SOLUBILITIES

Textile dyes are never of a high degree of purity. Some of the impurities are accidental; others are added intentionally so that dyers can obtain the desired shade without having to measure out dyes in very small quantities. Inasmuch as the early biological stains were textile dyes without much, if any, modification, it is natural that some of them should also have been of low dye content, and also that different batches should have been of various degrees of purity. In general the post-war dyes are much more pure than those available before the war. This makes it difficult to prepare stain solutions identical in strength with those prepared before the war.

There are two general types of stain formulae: in one a definite weight of dry dye is specified; in the other a certain volume of a saturated (generally alcoholic) solution of the dye. Each type of formula has its own possibilities of error; and to appreciate the problem it is necessary to understand certain facts in regard to the solubilities of dyes.

The error inherent in the first type of formula is plain at a glance. If two different staining solutions are made up containing 1 g. per 100 cc. of dry methylene blue, and in one case the actual dye content of the dry stain is 90 percent, while in the other only 55 percent (a difference actually observed in samples on the market), it is plain that the two solutions must differ greatly in their strength. For this reason an early recommendation of the Commission (1923b) was that formulae of the second type be preferred, on the assumption that a saturated solution of a dye would be more likely to be of constant dye content than different lots of dry stain bought in the market.

This recommendation, however, was made without complete understanding of the actual facts of the case. The amount of a dye that will go into solution in either water or alcohol depends upon the amount of mineral salts present. If a dye contains a large percentage of sodium chloride, for instance, a saturated solution will be of considerably lower actual dye content than if the dye were free or nearly free from salt; the sodium chloride prevents the solvent from taking up as much of the dye as it would normally. For this reason two staining solutions each containing 10 percent by volume of a saturated solution of the two methylene blues above mentioned would be quite different from each other in actual dye content, altho possibly more nearly alike than if they had been prepared with identical weights of the dry stain.

As soon as these facts were fully understood, the Commission (1923e) modified its recommendation. It is plain that the only way

two staining solutions can be made identical if different batches of stain are used is to make them up on the basis of the weight of *actual dye* present in the stain used. This can be done only if the manufacturer has co-operated to the extent of printing the actual dye content of each batch of stain on the container in which it is sold. This is not yet commonly done; but the Commission is issuing its certification only to batches of stain on which the total dye content is stated. In this way it is hoped that eventually all stains on the market will be so labeled; and then when staining formulae are readjusted so as to call for definite quantities of actual dye, the preparation of staining solutions will be put on a more scientific basis.

CHAPTER III

THE SPECTROPHOTOMETRIC ANALYSIS OF DYES.

DYES are extremely difficult to analyze by chemical methods. Their chemistry is in many cases obscure, they often differ from one another only in the way the chemical groups are combined together; different dyes may react alike to all known chemical tests and differ so slightly in solubility that it is difficult to distinguish one from another. For all these reasons it proves that spectrophotometric methods offer decided advantages in the examination of dyes over methods of chemical analysis, both in respect to general utility and in regard to convenience of application.

When a ray of light passes thru a prism it is resolved, as is well known, into many rays differing from each other in wave length and in color. Now the color of any substance arises from the selective absorption or reflection of definite parts of the visible spectrum, as light passes thru or is reflected from this substance. In the visible spectrum is included light of wave lengths intermediate between about 400 and 725 millimicrons. (The millimicron, denoted by the symbol $\mu\mu$, is one millionth of a millimeter in length.) The color of the light in the spectrum varies, with increasing wave length, from violet to red, appearing blue at about 450$\mu\mu$, green at about 500$\mu\mu$, yellow at about 550$\mu\mu$, and orange at about 600$\mu\mu$, as shown in Fig. 1. The color of light which reaches the eye after transmission thru or reflection from a colored substance is complementary to the color of the light absorbed by that substance. A violet dye, for example, appears violet because of its predominent absorption of yellow light. The complementary colors corresponding to the various parts of the spectrum are also shown in Fig. 1 beneath the colors of the spectrum.

The color of substances is ordinarily of complex origin, depending

upon the absorption of light in varying degrees, over an extensive spectral range. Whereas the unaided eye is able to register only the composite effect, it is possible to resolve this effect into its component factors with the aid of a spectrophotometer. Altho the eye is unable to distinguish between a violet dye and a suitable mixture of a red and a blue dye, the heterogeneous character of the mixture is readily apparent upon spectrophotometric examination. Pure dyes may have simple absorption spectra, in that their light absorption is all at one part of the spectrum, or they may be more complex, showing two or more points on the spectrum at each of which light is absorbed to greater extent than on either side of it. Thus even in the instance of pure products of identical color to the eye, the spectrophotometer frequently reveals decided differences when the character of the light absorption is considered in detail.

The essential principle of spectrophotometric analysis may be understood by reference to Fig. 2, which is a diagram of a spectrophotometer. Two parallel beams of light of equal intensity enter

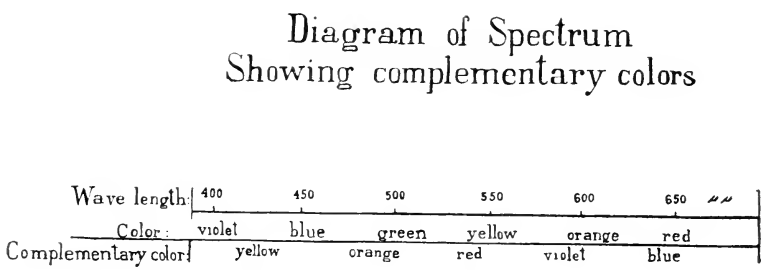


FIG. 1. Diagram of spectrum

the photometer box by separate orifices, pass thru a prism where they are resolved into visible spectra, and then reach the eye in contiguous fields so that very accurate comparison between the two spectra is possible. The arrangement is such that one beam passes directly to the prism whereas the intensity of the second beam may be reduced in any desired proportion by revolving the photometer circle. A glass cell containing a dilute solution of the dye to be examined is interposed in the path of the first beam and a similar cell containing water (or whatever solvent is used in the case of the dye) in the path of the second beam. The spectrum of the beam which has passed thru the dye solution will be found deficient in those portions which have been absorbed by the dye: and the degree of the deficiency at any position in the spectrum may be measured by determining the degree to which the intensity of the light of the second spectrum must be reduced in order to obtain an equal intensity in the two fields observed by the eye.

The shutter of the eyepiece may be partially closed so that only a narrow spectral range is visible; this allows the eye to concentrate on the matching of two small fields, each of which appears uniform in color. The instrument is provided with a screw drum, calibrated in wave lengths, by means of which the prism may be rotated in such a manner as to bring light of any desired wave length into the center of the field of vision.

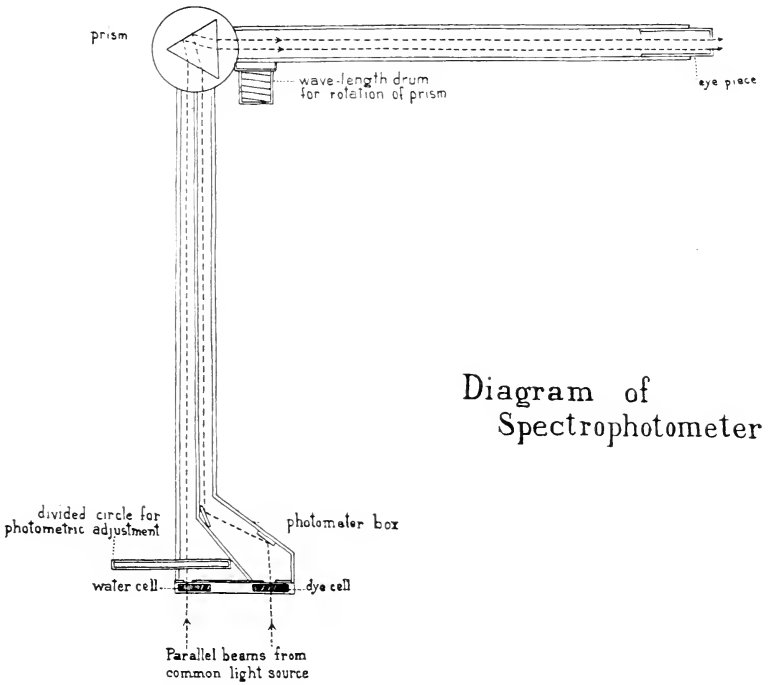


FIG. 2. Diagramatic section thru a spectrophotometer.

The photometer circle may be calibrated in various ways. In the general examination of dyes it is convenient to obtain the data in the terms of a factor known as the Bunsen extinction coefficient (E), and to employ a circle from which such values may be read directly.

In measuring the complete visible absorption of a dye, a series of measurements is made over the portion of the spectrum in which any appreciable absorption may be noted. This is done by setting the drum at some definite wave length and observing whether both beams of light reaching the eye are of the same intensity; if not, the photometer circle is turned until the beam

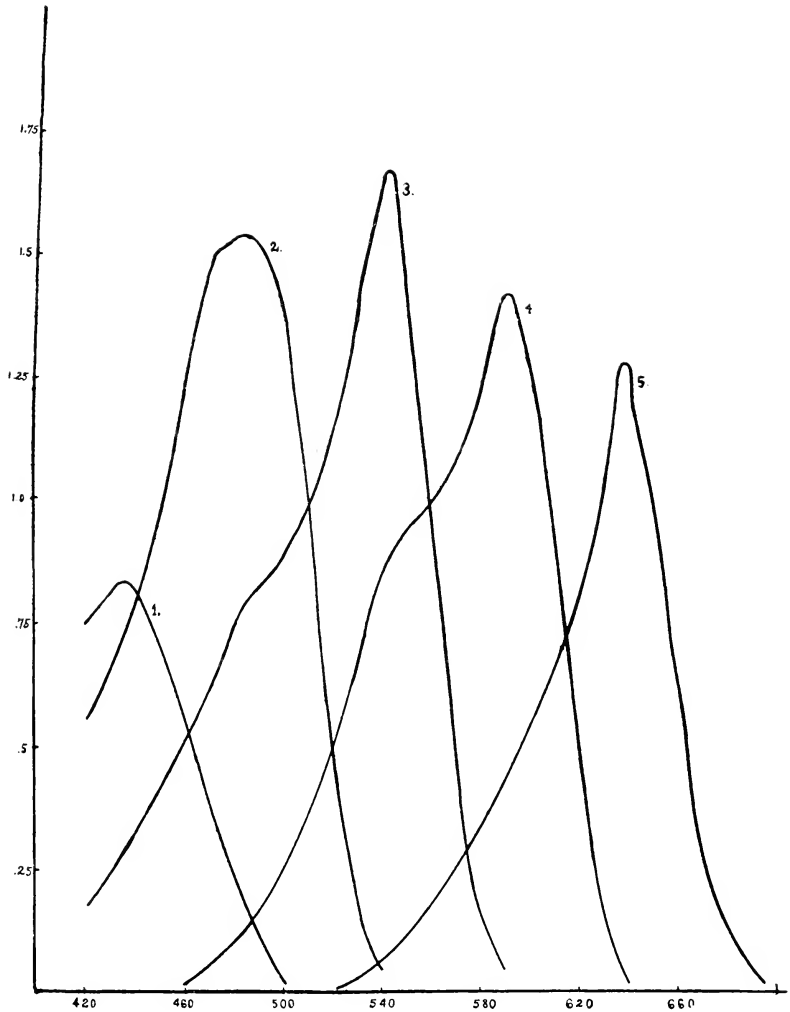


FIG. 3. Absorption curves of five dyes of different colors:

1. Tartrazine (yellow)
2. Orange G.
3. Fuchsin (red)
4. Crystal violet
5. Neptune blue BG.

which has not passed thru the dye is of the same intensity as that which has passed thru the dye cell. A reading of the extinction coefficient is then made. Further readings may be made at intervals of 10$\mu\mu$, with intermediate determinations in the immediate vicinity of the maximum absorption or at any other point at

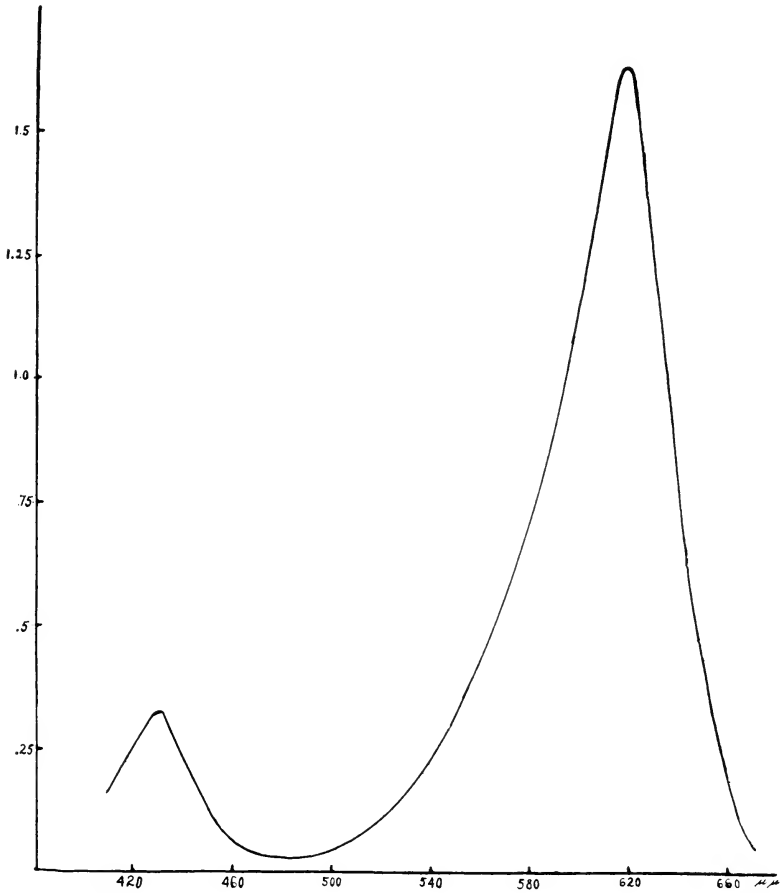


FIG. 4. Absorption curve of victoria green.

which it may appear desirable to bring out detail. If extinction coefficients are then plotted against wave length a graphic representation of the absorption band of the dye is obtained.

If measurements are carried out under suitable standardized conditions, the spectral position and the general form of the ab-

sorption curve are characteristic of the individual dye, while the magnitude of extinction coefficients (the height of the curve) varies directly with the amount of dye present. The absorption curves of dyes which are very closely related in structure are sometimes so similar as to be practically identical. In such instances the individual dyes may be recognized by means of quantitative determinations of the degree in which their absorption is modified under the influence of suitable variations in conditions.

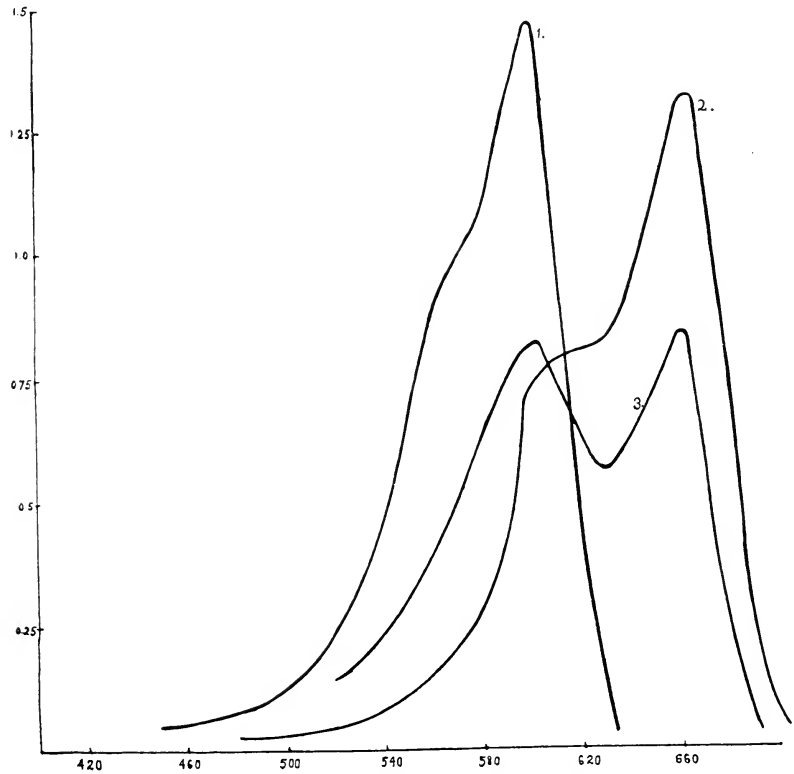


FIG. 5. Absorption curves of:

1. Thionin
2. Methylene blue.
3. Apparent mixture of these two dyes, incorrectly marketed (altho in good faith) as a dye intermediate between them in chemical composition.

The absorption curves of typical yellow, orange, red, violet, and blue dyes are recorded in Figure 3. It will be noted that their maximum absorption in each case falls within the range of the complementary color (cf. Fig. 1). The great majority of dyes of these

colors, in the usual solvents and under the usual conditions, show but one absorption band in the visible spectrum. The curves are seldom perfectly symmetrical, however, and usually give indications of localized secondary absorption in some portion of the band. It has been shown that this secondary absorption is due, in numerous instances, to a tautomeric form of the dye. It should never be accepted as evidence of the presence of a second dye unless it has been ascertained that it is not found with a pure sample of the dye under conditions of examination.

The absorption curve of a green dye is recorded in Fig. 4. It has a principal band in the red and a secondary band in the violet. Both the absorption curve and the color of the dye could be matched closely by mixing a suitable blue and a yellow dye in the correct proportions. All green dyes absorb appreciable amounts of violet light as well as of red light.

In Fig. 5 is given the absorption curve of a dye mixture, together with the curves of the component dyes. The mixture is reported to have been marketed in good faith as asymmetrical dimethyl thionin, a dye which is intermediate in constitution and in color between thionin and methylene blue (see methylene azure p. 48). The absorption curve plainly indicates the presence of two dyes, and suggests their probable identity. (It would be advisable to effect the separation of small amounts of both dyes, if their positive identification is desired.) The color of the mixture is very similar to that of dimethyl thionin. The absorption curve of that dye, however, is a simple and well defined curve resembling those of thionin and methylene blue, but occupying an intermediate position in the spectrum.

This illustration shows how valuable the spectrophotometric analysis may be in determining whether a given product is a simple dye or a mixture of two or more dyes. This fact, together with its use in determining the exact shade of any dye, makes it the most valuable test to apply to a stain, other than to determine by actual use whether the sample will prove satisfactory to the microscopist or not.

CHAPTER IV

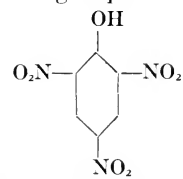
DYES OF THE NITRO, AZO, AND OXYQUINONE GROUPS

1. THE NITRO GROUP

In this group the chromophore is $-NO_2$. The chromophore is of such a strongly acid character that the dyes of this group are all acid dyes. The best known nitro dye is picric acid.

PICRIC ACID C. I. NO. 7*

Picric acid is formed by the action of nitric acid on phenol, thus introducing three nitro groups:



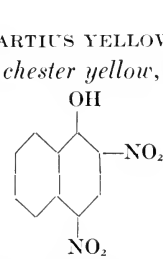
(*An acid dye; absorption maximum about 360 in alcohol*)

This compound forms salts by the dissociation of the-OH group, and the salts have considerable value as stains. Ammonium picrate is the one most commonly thus used.

Picric acid (or one of its salts) is quite extensively employed in contrast to acid fuchsin in the VanGieson connective tissue stain. It is also used as a general cytoplasmic stain in contrast to the basic dyes. It has further application as a fixative for tissues that are to be sectioned.†

MARTIUS YELLOW C. I. NO. 9

Synonyms: *Manchester yellow*, *Naphthol yellow*.



(*An acid dye; absorption maxima about 445, [399, 379]*)

Martius yellow has been used by Pianese in combination with malachite green and acid fuchsin for studying cancer tissue; the same technic was applied to plant tissue by Müller, and is now quite

*This abbreviation stands for the number in the "Colour Index"; see Chapter II, p. 23.

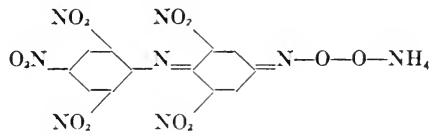
†For bibliographic references concerning the procedures referred to in this chapter see Table 2 in Appendix I, pp. 110–128, and also the bibliography in Appendix III, p. 138.

extensively used by plant pathologists in studying sections of tissue infected by fungi. The dye is also used in preparing certain light filters used in photomicrography.

AURANTIA C. I. NO. 12

Synonym: *Imperial yellow.*

This dye is the ammonium salt of hexanitro-diphenylamine.

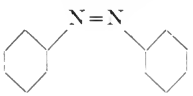


(*An acid dye; absorption maximum about 425*)

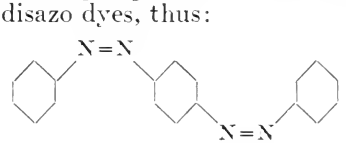
It is obsolete as a textile dye and is almost unknown as a biological stain. It is called for, however, in combination with toluidine blue and acid fuchsin in the Champy-Kull technic for demonstrating certain cell constituents (mitochondria, etc.)

2. THE AZO GROUP

The azo dyes are characterized by the chromophore —N=N— joining benzene or naphthalene rings, thus:

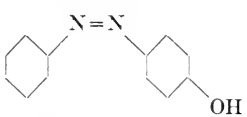


It is possible for the azo group to occur more than once in a molecule, forming the disazo dyes, thus:



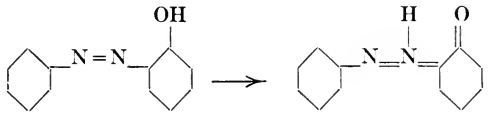
The azo chromophore is distinctly basic; but not sufficiently so to make the dyes basic when they contain hydroxyl radicals. Those containing amidogen radicals are, of course, pronouncedly basic.

The position of the hydroxyl or amidogen group on a benzene ring in relation to the azo group is important. Ordinarily they are in the para position to each other, thus:

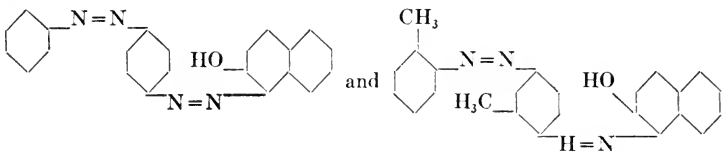


The ortho position is next frequently assumed; rarely the meta position. When the hydroxyl group assumes the ortho position the character of the compound is quite distinct from that of the para

compounds. By a rearrangement of the atoms such a compound is sure to change to a quinoid form, thus:



A compound of this latter structure cannot form salts and does not act as an ordinary dye. It does, however, prove to be soluble in oil and is able to color it by an apparently physical process. Hence the azo-ortho-phenols, or azo-beta-naphthols, like Sudan III and Sudan IV,

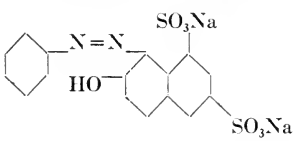


are important fat staining dyes.

ORANGE G. C. I. NO. 27

Synonym: *Wool orange 2G.*

Slightly different grade: *Orange GG, GMP.*

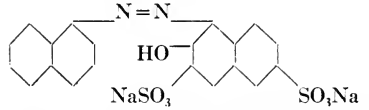


(*An acid dye; absorption maximum about 485*)

This dye is strongly acid because of the two sulphonic groups. It is one of the most valuable plasma stains in histological work. It has great use as a background stain for haematoxylin and other nuclear dyes in cytology. It is frequently employed, both by botanists and zoologists, as a cytoplasmic stain, together with the two nuclear dyes safranin and gential violet in the Flemming triple stain. It is of importance to the pathologist for its use with anilin blue and acid fuchsin in the Mallory connective tissue stain; and is used in various other double and triple staining methods, such as that of Ehrlich-Biondi-Heidenhain, in which it is mixed with methyl green and acid fuchsin. The Erhlich "triacid mixture," also a combination of these same three dyes, is used in staining blood. A further use is Bensley's "neutral gentian," a combination of orange G and gential violet for staining the islands of Langerhans.

Synonyms: *Fast red B* or *P. Cerasin. Archelline 2B. Azo-bordeaux. Acid bordeaux.*

Various grades denoted: *Bordeaux B, BL, G, R extra.*



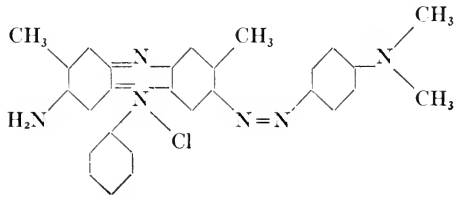
(*An acid dye; absorption maximum about 520*)

Bordeaux red is used as a cytoplasmic stain, in particular when Heidenhain's haematoxylin is to be used immediately afterward as a nuclear stain. It has also been used by Gråberg with thionin and methyl green for staining sections, particularly of spleen, testis, and liver.

JANUS GREEN B. C. I. NO. 133

Synonym: *Diazin green.*

This is an azo dye having an azin as well as an azo chromophore group, and is thus related to the safranins. It is a compound of diethyl safranin with dimethyl anilin thru an azo group.



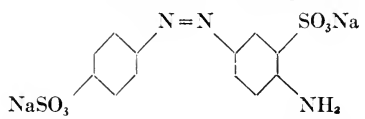
(*A basic dye; absorption maximum about 592.7*)

Janus green is best known for its use in demonstrating chondriosomes, stained *intra vitam*, according to the technic of Michaelis, and as more recently developed by Cowdry and Bensley. It is also used by Faris with neutral red for sections of embryos.

FAST YELLOW C. I. NO. 16

(*Echt Gelb*)

Synonym: *Acid yellow.*

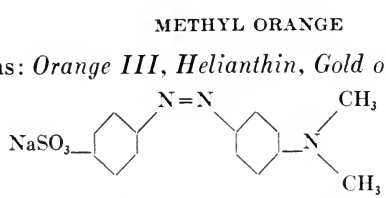


(*An acid dye; absorption maximum about 490 in acid solution*)

This dye is rarely used as a biological stain, but is called for by Schaffer for staining sections of bone, and by Unna in certain stain mixtures used in studying the phenomenon called by him chromolysis.

METHYL ORANGE C. I. NO. 142

Synomyms: *Orange III*, *Helianthin*, *Gold orange*, *Trapaeolin D.*

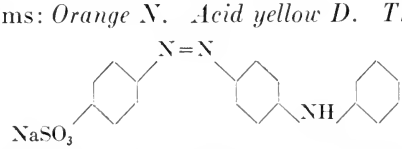


(*A weakly acid dye; absorption maximum about 506 in acid solution*)

This dye has little use as a stain, but is widely employed as an indicator, as it is red in acid, and orange in alkaline solutions. Its chief value as an indicator is that it is sensitive to mineral acids without being affected by carbonates or most organic acids. It has been used by Bergonzini in the place of orange G in the Ehrlich-Biondi stain; and by Ebbinghaus for staining keratin in sections of skin.

ORANGE IV. C. I. NO. 143

Synonyms: *Orange N. Acid yellow D. Tropaeolin OO.*

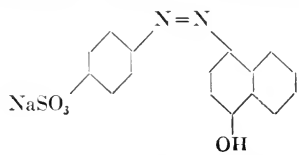


(*An acid dye; absorption maximum about 527 in acid solution*)

The only biological use of this dye seems to be occasionally as an indicator.

ORANGE I. C. I. NO. 150

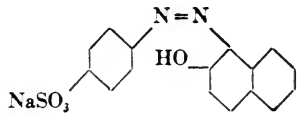
Synonyms: *Naphthol orange. Tropaeolin G.* or *OOO No. 1.*



(*An acid dye; absorption maximum about 476*)

This is another dye which is turned red by excess of alkali and has therefore some use as an indicator.

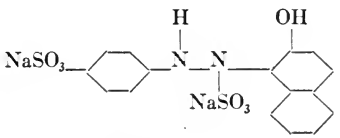
Synonyms: *Gold orange. Orange A, P,* or *R. Acid orange. Orange extra. Mandarin G. Tropaeolin OOO No. 2.*



(*An acid dye; absorption maximum about 490*)

This dye, which differs from Orange I only in the position of the hydroxyl group on the naphthalene radical, is similar to it in color and properties, but does not change color with changing reaction of its solution.

NARCEIN C. I. NO. 152

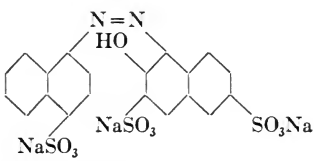


(*An acid dye*)

This dye is a derivative of Orange II, prepared from the latter by treatment with sodium bisulfite. It is rarely used either as a textile dye or in microscopic technic. It has been called for by Ehrlich, however, in combination with pyronin and methyl green or methylene blue to form a neutral dye.

AMARANTH C. I. NO. 184

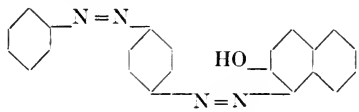
Synonyms: *Naphthol red. Fast red. Bordeaux. Bordeaux SF. Victoria rubin. Azo rubin. Wool red.*



(*An acid dye; absorption maximum about 525*)

Amaranth is not a commonly used stain, but is of considerable importance as a food color. It has been used by Griesbach for staining axis cylinders.

Synonyms: *Sudan G. Tony red. Scarlet G or B. Fettponceau G. Oil red. Cerasin red.*



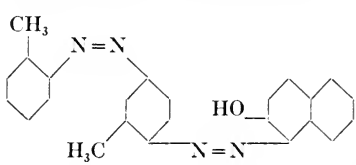
(*A weakly acid dye; absorption maximum about 641, [590]*)

In this dye the hydroxyl group is in the ortho position with respect to the azo group. As explained above (p. 34), such compounds show a tendency toward intramolecular rearrangement so that the hydrogen atom detaches itself from the hydroxyl group and becomes fixed to the neighboring nitrogen. Such a compound is neither acid nor basic, and not being able to form salts is not an ordinary dye, but is fat soluble and has the power of coloring fat. This fact gives Sudan III its chief value to the histologist. It was introduced as a fat stain by Daddi in 1896.

For some time Sudan III was the only important fat stain known. More is now known in regard to fat soluble stains, thanks to the research of Michaelis (1901). It was he who showed the relation of this property of certain dyes to their lack of basic or acid character. He showed that new dyes with this property and of greater staining power might be built up synthetically by taking advantage of the fact that the azo group will attach itself in the ortho position if the para position is already occupied. In this way azo-ortho-phenols and beta-naphthols can be prepared, and they prove to be fat soluble. Michaelis suggested the following dye, which has now to a considerable extent replaced Sudan III.

SUDAN IV C. I. NO. 258

Synonyms: *Scarlet red. Scharlach R. Oil red IV. Fettponceau. Ponceau 3B.*



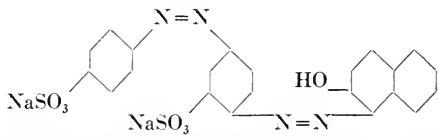
(*A weakly acid dye; absorption maximum about 657.4, [605.5] in H_2SO_4*)

This di-azo naphthalene compound is similar to Sudan III except that it is a dimethyl derivative. This fact makes it a deeper, more intense stain; but having the hydroxyl group in the ortho

position, it has similar physical properties and is fat soluble. It is, therefore, one of the best fat stains known.

BIEBRICH SCARLET, WATER SOLUBLE C. I. NO. 280

Synonyms: *Croceine scarlet. Scarlet B. or EC. Ponceau B. Double scarlet.*



(*An acid dye; absorption maximum about 503.5*)

The chief biological application of this dye is for medicinal purposes, but it is occasionally used as a plasma stain, notably for tissues after staining with polychrome methylene blue or Unna's haematein. It has also been made use of by Paladino mixed with alum haematoxylin for double staining effect on histological material.

BISMARCK BROWN Y C. I. NO. 331

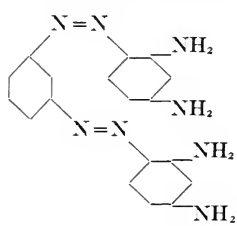
Synonyms: *Vesuvin. Phenylene brown. Manchester brown. Excelsior brown. Leather brown.*

Slightly different shade: *Bismarck brown G.*

To be distinguished from: Bismark brown R or GOOO (C. I. No. 332.)

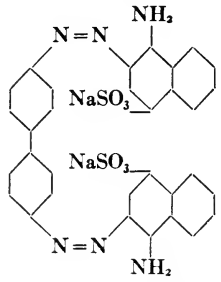
(*An acid dye.*)

The various shades of Bismarck brown are mixtures of different compounds, the most important of which are salts of the following:



This dye was formerly employed quite extensively as a contrast stain, but has now been replaced to some extent by others. It is still used, however, as a mucin stain, and is good for vital staining and for staining in bulk. It is employed in staining cellulose walls of plants in contrast to haematoxylin; and occasionally for staining bacteria in contrast to gential violet in the Gram technic.

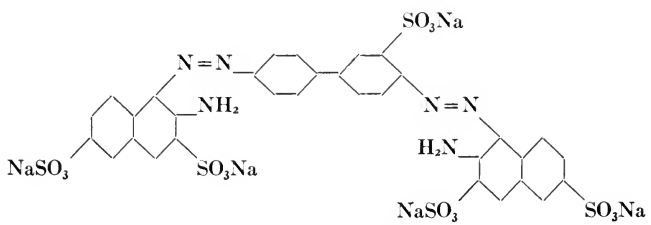
Synonyms: *Congo. Cotton red. Direct red.*



(*An acid dye; absorption maximum about 485.*)

This dye is best known to the biologist as an indicator. The dye acid is blue, but its sodium salt is red. The red color of the salt is readily changed by weak acids into blue. Besides serving as an indicator, congo red has certain histological uses, as for axis cylinders (Griesbach) for embryo sections (Schaffer), for staining plant mucin, and as a general background stain in contrast to haematoxylin and other nuclear dyes. It has been used by Klebs as a reagent for cellulose.

TRYPAN RED C. I. NO. 438

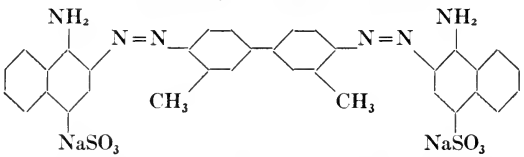


(*An acid dye.*)

The chief use of this dye is as a vital stain.

BENZOPURPIN 4B C. I. NO. 448

Synonyms: *Cotton red 4B. Dianil red 4C. Diamin red 4B. Sultan 4B. Direct red 4B.*

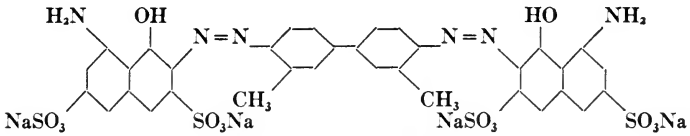


(*An acid dye; absorption maximum about 497.*)

This dye has also been used for vital staining; and has been employed by Zschokke as a plasma stain especially in contrast to haematoxylin.

TRYPAN BLUE C. I. NO. 477

Synonyms: *Chlorazol blue 3B. Benzo blue 3B. Dianil blue H3G. Congo blue 3B. Naphthamine blue 3BX. Benzamine blue 3B. Azidine blue 3B. Niagara blue 3B.*



(*An acid dye.*)

Apparently the only biological use of this dye is in vital staining.

Other azo dyes sometimes mentioned in connection with histology are:

Janus red; C. I. No. 266

Tropaeolin O; C. I. No. 148. Syn: *Chrysoin. Gold yellow. Acid yellow*

Tropaeolin Y; C. I. No. 148 (see note).

Roccellin; C. I. No. 176. Syn: *Fast red A, AV*, or *O. Cerasin, Rubidin. Cardinal red.*

Crystal ponceau 6R; C. I. No. 89; Syn: *Ponceau 6R.*

Carmin naphtha; C. I. No. 24. Syn: *Sudan 8. Scharlach B. Oil yellow.*

Alizarin yellow GG; C. I. No. 36. Syn: *Anthracene yellow. Benzene yellow.*

Chrysoidin R; C. I. No. 21. Syn: *Cotton orange. Cerotin orange.*

Chrysoidin Y; C. I. No. 20. Syn: *Brown salt R. Dark brown salt R.*

Alizarin yellow R; C. I. No. 40. *Alizarin orange. Benzene yellow PN. Orange R; Anthracene yellow RN.*

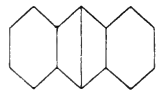
Diamond flavine; C. I. No. 110.

Diamond black F; C. I. No. 299. Syn.: *Salicin black. Chrome black.*

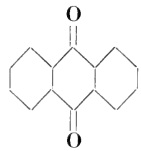
Niagara blue 4B; C. I. No. 520. Syn.: *Niagara sky blue. Benzoin sky blue. Dianil blue H 6 G. Congo sky blue. Naphthamine blue.*

3. THE OXYQUINONE GROUP

The oxyquinone dyes include derivatives of anthracene,

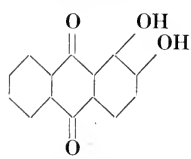


thru its oxidation product anthraquinone:



These dyes are the first to be considered here in which the quinoid structure occurs. The quinoid ring, which is the most important chromophore in nearly all the dyes to be discussed in the three following chapters, forms very strong chromogens, which require only the addition of auxochrome groups to be converted into strong dyes, either basic or acid. The chromogen anthraquinone is converted into a dye by the addition of hydroxyl groups, its best known derivatives among the dyes being: 1:2 dihydroxy-anthraquinone (alizarin) and 1:2:4 trihydroxy-anthraquinone (purpurin). Both of these compounds occur in nature in the root of madder, being the colored principles of madder extract. They have the property of combining with metalic oxides to form so-called "lakes", insoluble compounds of different color from the dye entering into them. This makes them valuable ones to use after mordanting with aluminium, iron or chromium compounds.

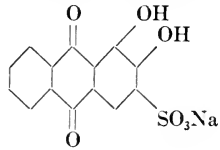
ALIZARIN C. I. NO. 1027



(*An acid dye; absorption maxima about* [*610.8*], *566.5*, [*527.6*] *in alkaline solution.*)

Alizarin stains tissues a feeble yellowish red if used on them directly. In the presence of aluminium compounds intense red colors are formed; bluish violet in the presence of iron; and brownish violet in the presence of chromium. It has been used as a stain for nervous tissue. The chief present use of alizarin, however, is as an indicator.

Synonyms: *Alizarin red, water soluble. Alizarin carmin. Alizarin sulphate.*

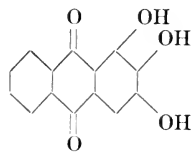


(*An acid dye.*)

This dye, sodium alizarin sulphonate, is used by Benda for staining chromatin in combination with crystal violet, the chromatin staining brown, while the mitochondria stain violet. It is also used as a vital stain for nervous tissue in small invertebrates, and by Schrötter for sections of nervous tissue.

PURPURIN C. I. NO. 1037

Synonyms: *Alizarin No. 6. Alizarinpurpurin.*



(*An acid dye; absorption maxima about* [*521.1*], *485.5*, [*455.5*] *in alcohol.*)

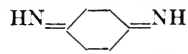
Purpurin is very similar to alizarin, but forms scarlet red lakes with alumina. It has been used as a nuclear stain for histological material, and for determining the presence of insoluble calcium salts in the cell contents.

CHAPTER V

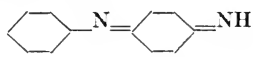
THE QUINONE-IMIDE DYES

THE dyes of the quinone-imide group contain two chromophore groups, the indamin group —N=, and the quinoid benzene ring

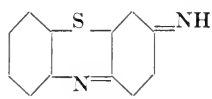
They are derivatives of the theoretical compound paraquinone imide, which, if it existed in its free state, would have the formula



In the typical indamin formula one of the imide hydrogen atoms is replaced by a phenyl group, thus:

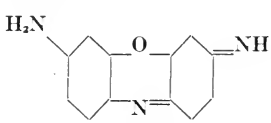


In the thiazins the introduction of a sulfur atom, attached to both the phenyl and the quinone groups, forms a third closed ring, as:



imido-thio-diphenylimide

In the oxazins, an oxygen atom takes the place of the sulfur of the thiazins, thus:



oxazin

1. THE INDAMINS

No dye in this group is a common biological stain. The following are occasionally mentioned, however, in connection with histology:

Bindschedler's green. A tetramethyl indamin. C. I. No. 819.

Toluylene blue. A diamido, dimethyl indamin. C. I. No. 820.

2. THE THIAZINS

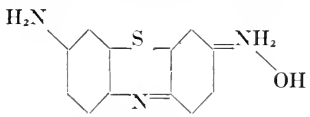
The thiazins constitute one of the most important groups of dyes from the standpoint of the biologist; while for textile dying the group contains but a small number of dyes of any importance. In these compounds, as mentioned above, the two benzene rings are further joined by a sulfur atom.

Synonym: *Lauth's violet.*

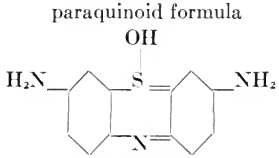
*(A basic dye; absorption maximum about 602.)**

Thionin, having two amino groups, is a strongly basic dye. The exact structural formulae of this dye and its derivatives, as well as many others in which two benzene rings are similarly joined, are in some dispute. At least two types of formulae are possible for the thiazins and oxazins, as well as for the xanthene dyes (Chapter VII). One type is known as the orthoquinoid, the other as the paraquinoid.

It will be recalled (see p. 13) that when the quinoid ring is formed the two hydrogen atoms replaced by atoms or groups with double valency bonds may be either in the para or in the ortho position to each other. It will also be recalled from elementary chemistry that sulfur and oxygen may be either bivalent or tetravalent. These facts make it possible for a thiazin or an oxazin to have either one or the other of the different structures represented by the following two formulae for the theoretical thionin base:



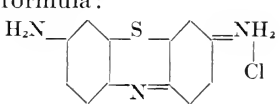
paraquinoid formula



orthoquinoid formula

In the case of the paraquinoid formula the compound is an ammonium base of the type discussed on p. 15, which is capable of salt formation thru its pentavalent nitrogen. In the case of the orthoquinoid formula the salt formation takes place thru the tetravalent sulfur, the base being of the type known as a sulfonium base. There are arguments in favor of either formula, and from the standpoint of the biologist it does not matter which is preferred. Possibly both forms actually exist simultaneously. For the sake of uniformity the paraquinoid form will be shown in the following pages wherever possible; but with the understanding that the orthoquinoid form is equally permissable.

The dye, thionin, is a salt, generally a chloride, of the above mentioned base; and on the assumption of paraquinoid structure, it has the following formula:



*See Fig. 5. p. 30.

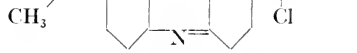
It is no longer used as a textile dye, and is very carefully to be distinguished from thionin blue (C. I. No. 926) which is known to the trade and is sometimes furnished in place of the desired dye when thionin is ordered. Thionin is an especially valuable dye for histological work on account of its metachromatic properties, that is its ability to impart different colors to different histological or cytological structures. It is a very valuable chromatin and mucin stain, proving especially useful in staining the tissue of insects; and is recommended by Ehrlich because it stains amyloid blue but mast cells and mucin red. It is a useful vital stain. Perhaps its greatest value at the present time is in the staining of frozen sections of fresh animal or human tissue, particularly in the study of tumors. It is also used by Frost for staining very young bacterial colonies in his "little plate" technic for counting bacteria. (Unfortunately Frost specifies thionin blue in one of his papers, altho the latter proves entirely unsatisfactory for the purpose.)*

METHYLENE BLUE C. I. NO. 922

Synonym: *Swiss blue.*

Various grades denoted: *Methylene blue BX, B, BG, BB;* grade preferred for biological work: METHYLENE BLUE MED. U. S. P.

Methylene blue is a salt of tetramethyl thionin (generally a chloride, altho other salts are known, such as sulfates). On the assumption of the paraquinoid structure, it has the formula:



(*A basic dye; absorption maximum about 665.*)

According to the general rule as to the influence of methylation on color it is less red in shade than thionin and is therefore a purer blue. Its absorption curve has a maximum at about 665$\mu\mu$, with a lesser peak at about 610$\mu\mu$. The methylene blue of commerce is generally a double salt, the chloride of zinc and methylene blue. The zinc is toxic, however; so for some time the zinc-free methylene blue chloride has been prescribed for medicinal purposes; hence the meaning of the term Methylene blue Med. U. S. P. The zinc double salt is less soluble, particularly in alcohol, so for most staining purposes is less desirable. The investigations of the Commission show that for all ordinary staining purposes the zinc-free compound is best; so that is the form at present recommended.

*For bibliographic references concerning the procedures referred to in this chapter, see Table 2 in Appendix I, pp. 110–128, and also the bibliography in Appendix III, p. 138.

Methylene blue is perhaps the stain which the pathologist and bacteriologist would have the greatest difficulty in doing without, and it is of great value to the zoologist as well. It is employed for a greater variety of purposes than any other biological stain except possibly haematoxylin; and for this reason was the first dye to be given a thoro investigation by the Commission. It is used: first, as a nuclear stain in histology, for which purpose its strongly basic character as well as the ease with which it can be applied without over-staining, make it quite valuable; secondly, as a bacterial stain, notably in milk work and in the diagnosis of diphtheria, where it is especially useful because it has an affinity for the bacterial protoplasm as great as that of the rosanilin dyes, but is less intense, more selective in its action and more subject to differentiation; thirdly in the vital staining of nervous tissue, where a non-toxic, basic dye is needed; fourth, in combination with eosin in the blood stains, thanks to the ease with which it can be partly converted into other dyes like methylene violet and methylene azure, and thus acquire polychrome properties; and lastly as an indicator in the Levine eosin-methylene-blue medium for differentiating the colon and aerogenes organisms.

The polychrome properties just mentioned are quite likely to develop in a methylene blue solution upon standing. Anyone who has had much experience with the stain is familiar with the occasional green tones from methylene green, the reddish shades of methylene azure (azure I) and methylene violet. Such a solution is known as "polychrome methylene blue." Its formation is hastened by boiling with alkali. In preparing blood stains the methylene blue solution is treated for this purpose with sodium carbonate, and then eosin is added, which enters into chemical combination with the other dyes present, inasmuch as eosin is an acid dye while methylene blue and its derivatives are basic. The combination of eosin and methylene blue is often spoken of as the eosinate of methylene blue. (For a more detailed discussion of the subject see Chapter VIII.)

It can be readily understood that an especially pure product is needed when the dye is to be used for vital staining or in blood work. For vital staining the U. S. P. zinc-free dye is always recommended, sometimes with even further purification; altho the recent investigations carried on by the Commission indicate that the U. S. P. product is sufficiently pure. For blood work there is frequently recommended a "methylene blue rectified for blood stains." This grade, however, is generally less pure than the medicinal or U. S. P. grade, and there seems no reason for specifying it. The same is true of various other grades such as those denoted BX, BG, etc., which are ordinarily purer than the textile dye, but less pure than the medicinal grade.

In a recent paper by Scott and French (1924b) it is claimed that the specially desired staining properties of methylene blue are

associated with the presence of lower homologs, particularly the dimethyl thionins. One of the dimethyl thionins is methylene azure A; hence the statement of Scott and French is merely another way of saying that methylene blue should be partially polychromed in order to have its best staining powers. These lower homologs are generally present to some extent in methylene blue as suggested by the minor peak in the absorption curve at 610$\mu\mu$. Scott and French show that a methylene blue may be specially prepared which contains more than usual of these lower homologs and that it is better than the ordinary product for their purposes. How widely their results can be applied to all microscopic uses of methylene blue cannot be decided at present. It is interesting that one particular instance has come to the attention of the Commission in which a pure methylene blue was unsatisfactory for a certain neurological procedure, but a crude textile methylene blue proved satisfactory. This may possibly have been due to the presence of other dyes in the impure sample.

One serious bit of confusion has arisen from the designation "methylene blue for bacilli" which was used on a certain type of methylene blue imported before the war. This was the label placed on a certain type of zinc salt, containing a small amount of free chloride. Its designation seemed to imply that it was especially adapted for staining bacteria; recent investigations indicate that it should rather be considered not good enough for any other purpose! Even for staining bacteria it is not especially satisfactory; for the most common methylene blue solution of the bacteriologist is the Loeffler formula, in which a certain amount of saturated alcoholic solution is used as a stock. Now, since the zinc salt is nearly insoluble in alcohol, such a stock solution contains little but the free methylene blue chloride present. For all these reasons the discontinuing of this grade of methylene blue is decidedly to be recommended.

METHYLENE AZURE*

Synonyms: *Azure I. Azure A. Azure B.*

This is one of the components of polychrome methylene blue, first described by Bernthsen (1885). Definite knowledge of its chemical nature was gained by Kehrmann (1906) and Bernthsen (1906). The former showed that there are two azures, the asymmetric dimethyl thionin, Azure A, and trimethyl thionin, Azure B. The symmetrical dimethyl thionin, which he prepared, was found to belong in a quite different category.

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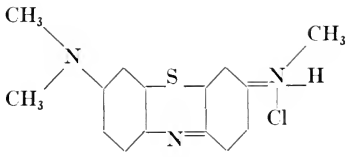
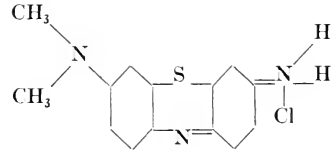
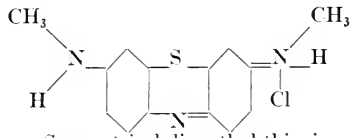
*For the following statements in regard to methylene azure and methylene violet the author is indebted to Dr. W. J. MacNeal.



Tri-methyl thionin
(Azure B, Kehrmann)



Asymmetric di-methyl-thionin
(Azure A, Kehrmann)



Symmetrical di-methyl thionin
(not an azure dye)

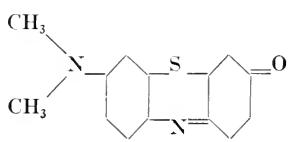
Bernthsen (1906) and MacNeal (1906) simultaneously and independently described the easy method of preparing azure by oxidizing methylene blue by chromate in acid solution and MacNeal (1925) has perfected this method so that it is now possible to obtain large yields of Azure A and a fair yield of Azure B.

Azure I (Giemsa) is a trade name applied to a secret preparation which appears to be a somewhat variable mixture of Azure A and Azure B. Azure II (Giemsa) is an intentional mixture of Azure I (Giemsa) with an equal quantity of methylene blue.

The azures are important constituents of all the polychrome methylene blue stains and are present in undetermined and variable amounts when these solutions are empirically prepared. For the preparation of the tetrachrome blood stain of MacNeal (see p. 90), a definite quantity of Azure A (Asymmetric di-methyl-thionin) is required.

METHYLENE VIOLET (Bernthsen)

Methylene violet is formed whenever methylene blue is heated with a fixed alkali or alkali carbonate. It is a feeble base with the formula

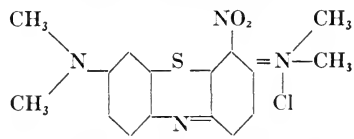


Its preparation from methylene blue is more difficult than that of Azure A. A fair yield (30 to 40 per cent) may be obtained by oxidizing methylene blue in dilute ammoniacal solution with potassium chromate and then driving off the ammonia by boiling with the addition of sodium carbonate. It may also be prepared from Azure A by boiling this with dilute alkali carbonate. Methylene violet precipitates out as needle crystals, insoluble in water. It

may be recrystallized from ethylene dichloride ($C_2H_4Cl_2$) in which it forms a deep carmine red solution. Although insoluble in water when pure, methylene violet is soluble when mixed with methylene blue or with the azures. It plays an important part in the nuclear and granule staining of the polychrome methylene blue stains. A definite quantity of this dye is employed in the tetrachrome blood stain of MacNeal.

Methylene violet (Bernthsen 1885) is not a textile dye and must not be confused with methylene violet RRA or 3RA, which is C. I. No. 842.

METHYLENE GREEN C. I. NO. 924



(*A basic dye; absorption maxima at about 660, 607.*)

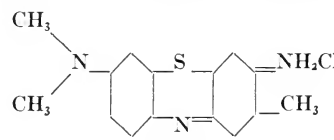
This dye is a mono-nitro methylene blue, obtained by the action of nitrous acid on methylene blue. The formula is probably as given above, but the exact position of the nitro group is uncertain.

It is occasionally used as a substitute for methyl green, especially by botanists in the case of wood and fixed chromatin, and gives good results in combination with eosin.

TOLUIDINE BLUE O C. I. NO. 925

Synonym: *Methylene blue O.*

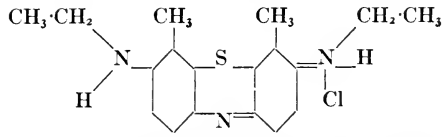
This dye is closely related to thionin and to methylene blue in structure, and even more closely to methylene azure A:



(*A basic dye; absorption maximum about 635.*)

Toluidine blue is not ordinarily used for textile dyeing, but is more easily prepared than thionin—a fact of considerable importance, as it has properties very much like the latter. It proves, in fact, that it can be substituted in many ways for thionin, as for example in staining frozen sections of fresh tissue. It is quite a useful stain, being an important ingredient of Pappenheim's panchrome stain for tissues and blood, and also the main constituent of the Albert stain, which is at present replacing methylene blue in the diagnosis of diphtheria.

Synonym: *Methylene blue NN.*



(*A basic dye; absorption maxima about* [*636.4*], *588.*)

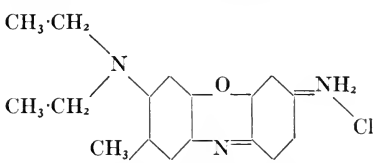
This dye has practically never been called for in microscopical work. The most interesting fact concerning it which has come to light relates to the VanWijhe technic as applied by Louise Smith (1920) for staining the cartilage of frogs. The latter specified methylene blue, but the results could not be duplicated with any domestic or foreign methylene blue subsequently obtained. When furnished thru the Commission with samples of various stains to try, it was found that her earlier results could be duplicated with new methylene blue—a fact which not only implies mislabeling of her original supply of methylene blue, but suggests that new methylene blue may have some value in histological work.

3. THE OXAZINS

This group is like the thiazins in chemical formula except that the sulfur atom is replaced by an oxygen atom. Only a few of the dyes find use in microscopic technic, and they are not stains having very general application.

BRILLIANT CRESYL BLUE C. I. NO. 877

Synonyms: *Cresyl blue 2RN* or *BBS; brilliant blue C.*

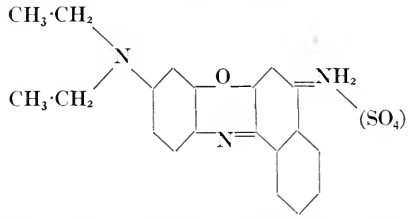


(*A basic dye; absorption maxima about 631.8* [*579.5*])

This dye is prized for certain special work on account of its highly metachromatic properties. Its chief biological use is for staining blood to bring out the platelets and the reticulated blood corpuscles.

Brilliant cresyl blue proved one of the most difficult stains to obtain in good quality since the war. The problem was finally solved however, and the pre-war stain has not only been equalled but surpassed.

Synonym: *Nile blue A.*



(*A basic dye; absorption maxima about 644.5, [592.2]*)

The use for which this dye is best known to the biologist is the Lorrain Smith fat stain. In this procedure the dye is boiled with dilute sulfuric acid, and thus hydrolyzed, with the introduction of oxygen in the place of the radical NH_2 $(SO_4)_{\frac{1}{2}}$, in other words producing a new dye of the class known as oxazones. This oxazone dye is red, and is fat-soluble. Nile blue sulfate itself, on the other hand, is not fat-soluble but combines readily with fatty acids. As a result the technic serves to distinguish between the free fatty acids in histological material and the neutralized fats, the former staining blue, the latter red.

Nile blue sulfate is also used unaltered for staining living tadpoles previous to making transplants, in order to distinguish the grafts.

CRESYL VIOLET

Synonym: *Cresylecht violet* (i.e., cresyl fast violet).

(*A basic dye; absorption maximum about 585.*)

No information is at hand concerning the exact chemical formula of this dye. It is understood to be a derivative of brilliant cresyl blue.

Cresyl violet is not a widely used stain, but finds some employment on account of its strongly metachromatic properties. It is valuable in making permanent preparations of nervous tissue. According to Ehrlich (1910, II, p. 78) it stains nuclei violet, plasma blue, amyloid, mucin and mast cell granules red. Williams (1923) uses it for staining sections of fresh tumor tissue.

As cresyl violet is not a textile dye, some difficulty has been found in obtaining it for biological purposes. Williams reports considerable trouble in this respect. Spectrophotometric examination of the pre-war material used by Williams shows it to have been a mixture, apparently of cresyl violet with another dye of more reddish cast. (See Ambler and Holmes 1924.) The domestic sample with which he obtained unsatisfactory results on account of its entire lack of metachromatic properties was this unknown red dye alone; while the domestic sample with which he obtained good results, altho not identical with those obtained with the pre-war

stain, was true cresyl violet without the reddish dye. True cresyl violet, like this last mentioned product can now be obtained in constant quality in America, and proves to have all the properties needed in this stain.

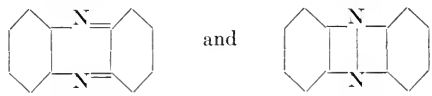
Other oxazin dyes sometimes mentioned in connection with histology are:

Capri Blue. C. I. No. 876.

Naphthol Blue. C. I. No. 909. Synonym: *New blue B, Fast blue 3R. Phenylene blue. Meldola's blue. Indin blue 2RD.*

4. THE AZINS

The dyes of the azin group are derivatives of phenazin, $C_6H_4{\cdot}N_2{\cdot}C_6H_4$, a compound containing two benzene rings linked thru two nitrogen atoms in such a way as to form a third ring. Two formula are possible:

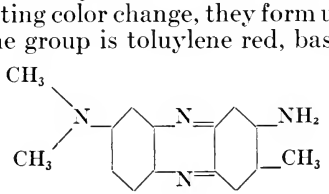


In the case of the first formula the quinoid ring is the chromophore; in the case of the second formula the azin group itself, $\begin{matrix}-N-\\ |\quad |\\ -N-\end{matrix}$, (see p. 17) is assumed to be the chromophore. The quinoid formula is generally preferred today.

Phenazin is weakly basic, but is not a dye as it does not contain auxochrome groups. In other words, it is a chromogen. Either an $-OH$ group or one or more $-NH_2$ groups may be introduced to give it dye properties. The acids and bases are very weak if there is only one auxochrome group present, and their salts are readily decomposed. For this reason some of them are of use as indicators. Strong bases are encountered only among the safranins where basic character is derived not only from the two $-NH_2$ groups but also from one of the azin nitrogen atoms which becomes pentavalent and takes part in salt formation.

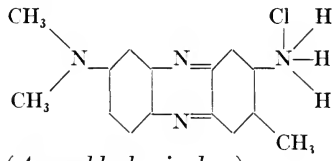
a. Amido-azins or Eurhodins

If one or more amino groups are introduced into a phenazin, a dye is formed of the class known as eurhodins. They are very weak bases, and therefore weak dyes; but as their salts are readily decomposed with a resulting color change, they form useful indicators. The best known of the group is toluylene red, base:



The chloride of toluylene red is the well known neutral red.

NEUTRAL RED C. I. NO. 825

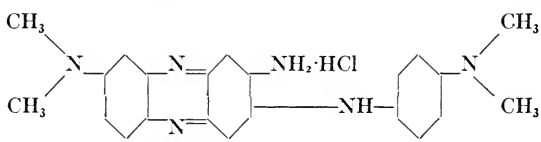


(*A weakly basic dye.*)

The name of this dye comes from its characteristic neutral color which is neither red or yellow. It is yellow in solutions a little below the neutral point (i.e., pH=7.0) in reaction and red in weak acids, even the reaction of ordinary tap water being sufficient to bring out the acid color; at a higher range of acid it turns blue. This gives it some value as an indicator. As an indicator it is also used in bacteriological media for distinguishing the colon from the typhoid organisms, and for recognizing other forms; altho it is employed for this purpose much less today now that other dyes have been shown to have even greater value for the same type of work.

As a stain it has special value where a weakly basic, non-toxic dye is called for. It is used as a vital nuclear stain; for the "vital" staining of blood, that is of fresh blood observed under a microscope in a moist chamber; and for staining fresh gonorrhoeal pus under similar conditions. It is used for bringing out the Nissl granules in nerve cells; it also has some use in general histological staining, especially for embriological tissue in combination with Janus green, as recommended by Faris.

NEUTRAL VIOLET C. I. NO. 826



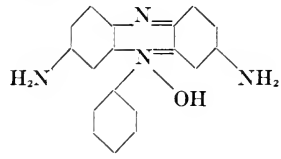
(*A weakly basic dye.*)

This dye is very similar in its properties to neutral red, except that, due to its greater molecular weight, it is more bluish, giving a violet instead of a red color. It can be used as an indicator, but has been seldom used in histology. Unna (1921) however, has recently used it in a dye mixture employed in the study of chromolysis.

b. Safranins

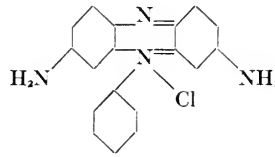
Quite a long series of azin dyes are known in which one of the nitrogen atoms of the azin group is pentavalent and another ben-

zene ring is attached to it. This pentavalent nitrogen allows the compounds to behave like ammonium bases; so with the amino groups which are always present, the basic properties of these dyes are very strong. The theoretical base of the simplest safranin would have the formula:



This form of ammonium base does not actually exist, as the safranin bases really occur in the form of anhydrides; but salts of these ammonium bases are the commonly known dyes. The commercial dyes are ordinarily chlorides.

There are two groups of safranins: the benzo-safranins in which the azin group unites two benzene rings; and the naphtho-safranins in which it unites two naphthalene groups. The simplest safranin is pheno-safranin, which is the chloride of the theoretical base just given, namely:



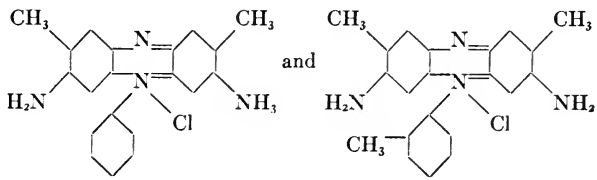
The commercial safranins are ordinarily methyl or ethyl substitution products of this; or occasionally phenyl substitution products. The one of greatest value to the biologist is generally called Safranin O.

SAFRANIN O C. I. NO. 841

Slightly different shades: *Safranin AG, T, MP, Y,* and *G.* (Altho all included in C. I. No. 841 they are different from the grade here described.)

(*A basic dye; absorption maximum about 515.*)

The common safranins of commerce, under various shade designations, are mixtures of di-methyl and tri-methyl pheno-safranin:



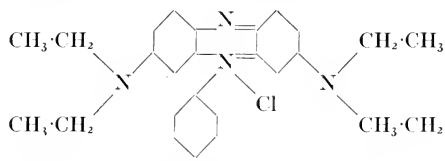
The shade differs according to the proportion of these compounds present, the red being deeper according to the proportion of the tri-methyl compound in the mixture. The type safranin O, which proves best for ordinary biological purposes, can be defined as having its absorption maximum at 515$\mu\mu$.

Safranin O is one of the most important nuclear stains known to the histologist. The botanist finds it especially valuable, as it brings out lignified and cutinized tissues in vascular plants, and can be employed in combination with a variety of contrast stains; it is valuable as a protein stain in plants, and can be used to stain spore coats. The cytologist makes use of it in the Benda technic to stain chromatin in combination with light green as a contrast stain; and even more widely in the Flemming triple stain, in which it is employed as a chromatin stain, together with gentian violet and orange G. The bacteriologist has some use for it, especially as a counterstain in the Gram technic (see p. 68).

AMETHYST VIOLET C. I. NO. 847

Synonyms: *Heliotrope B, Iris violet.*

This dye is tetra-ethyl pheno-safranin:



(*A basic dye; absorption maxima about 589, [545.5]*)

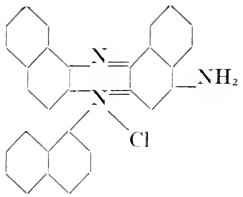
Amethyst violet has been used by Ehrlich and Lazarus as a basic dye in certain triple staining technics.

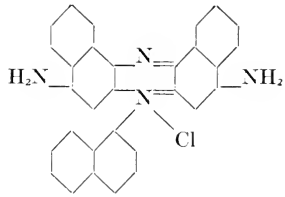
A further dye of this group which the biologist must take into account, altho it seems to have no significance as a stain, is methylene violet RRA or 3RA, C. I. No. 842 (syn.: *fuchsin* or *safranin extra blue.*) This dye is a di-methyl safranin in which the methyl groups are introduced into one of the amino groups instead of directly into the benzene ring. It has no connection with the methylene violet of Bernthsen, which is one of the constituents of polychrome methylene blue; see p. 49.

MAGDALA RED C. I. NO. 857

Synonyms: *Naphthaline red, naphthaline pink, naphthylamine pink, Sudan red.*

This is a naphtho-safranin, and is a mixture of the monamino and diamino compounds:



and


(*A basic dye; absorption maximum about 524.*)

A true Magdala red put on the market before the war under the name of Magdala red *echt* is very expensive. According to Chamberlain (1924 page 58) this is less satisfactory in botanical work than a cheaper form of Magdala red formerly available not labeled "echt." Chamberlain further states that he has been able to obtain recently results with phloxine identical with those which he used to be able to obtain with the less expensive form of Magdala red. A sample of the latter examined by the Commission proves apparently to be erythrosin,—in other words an acid dye of an entirely different group and very closely related to phloxine. This makes Dr. Chamberlain's failure to obtain results with Magdala red *echt* entirely comprehensible. (See also discussion under phloxine and erythrosin page 81–82.)

Magdala red is used by botanists with anilin blue, in staining algae. It was used by Flemming as a nuclear stain, and by Kultschitzky for staining elastic tissue.

c. THE INDULINS

Indulins are similar to safranins but are more complex: being quite highly phenylated amino derivatives. The only one to concern us is:

NIGROSIN, WATER SOLUBLE C. I. NO. 865

Synonyms: *Nigrosin W, WL*, etc. *Gray R, B, BB. Silver gray. Steel gray. Indulin black.*

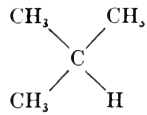
(*A basic dye; absorption maximum about 587.*)

The exact constitution of nigrosin is uncertain. It is recommended by Ehrlich for staining the tissue of the central nervous system either alone or in combination with other stains, and by Jarotsky for staining pancreatic tissue following haematoxylin. Botanists use it in studying algae and fungi. Pfitzer's picronigrosin serves as a chromatin stain. Nigrosin is also used by Unna in combination with "orange" (orange G?) in the study of the process of chromolysis.

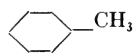
CHAPTER VI

THE PHENYL METHANE DYES

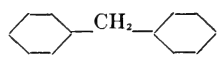
ONE of the most important groups of dyes, both from the standpoint of the dyer and from that of the biologist, is a group of substituted methanes, or in other words compounds with a central carbon atom. In methane, CH_4, it is possible to replace any of the hydrogen atoms with methyl, ethyl, or phenyl groups. If one H is replaced with CH_3, it becomes ethane, $CH_3 \cdot CH_3$. If two are replaced with CH_3 groups it becomes propane, $CH_3 \cdot CH_2 \cdot CH_3$; while if there are three substituent CH_3 groups it becomes iso-butane:



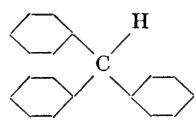
Similarly if one H is replaced with a phenyl group it becomes phenyl methane or toluene:



if with two it becomes di-phenyl methane:



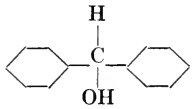
if with three it becomes tri-phenyl methane:



Certain substitution products of the di- and tri-phenyl methanes are among the most powerful dyes known.

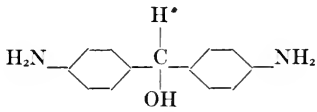
Di- and tri-phenyl methane, themselves, are not dyes, nor are they chromogens. They lack both the chromophore and the auxochrome groups. The first step (theoretically) in converting them into dyes is to introduce an -OH group in the place of one of the unsubstituted H atoms of the methane nucleus. The compound thus formed, which bears the same relation to the phenyl methane

as alcohol does to methane, is called a carbinol. A carbinol is methyl alcohol in which one or more of the hydrogen atoms may have been replaced with an alkyl radical or a benzene ring. Thus:

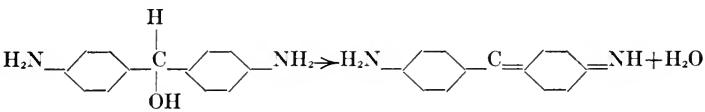


diphenyl carbinol

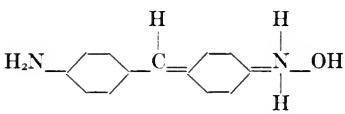
It is next theoretically possible to attach amino groups to the benzene rings. Thus in the case of di-phenyl carbinol it is possible to obtain di-amino di-phenyl carbinol:



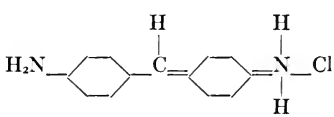
Now this latter compound contains the necessary auxochrome groups; but it is not yet a dye. No carbinol is a dye, because it lacks a chromophore group. The carbinols are important in dye chemistry, however, because upon dehydration a rearrangement of the bonds in the molecule takes place giving the quinoid benzene ring, which as we have seen is a powerful chromophore. Thus:



Now this latter compound is the anhydride of a true dye base. Upon hydration it should theoretically become:



Such a compound could exist only in watery solution. It is known only by its salts, the true dyes, as:



Altho the theoretical compound given above is the true dye base, the carbinols are often known as carbinol bases of the phenyl methane dyes or are sometimes called leuco-bases or color bases. They are not bases in the chemical sense, however, as they do not

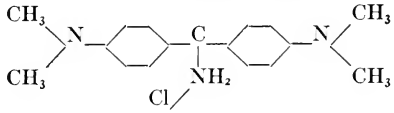
have basic properties. As stated above, they lack the chromophore group, and hence are colorless.

1. DI-PHENYL METHANE DERIVATIVES

The di-phenyl methanes are of practically no biological significance. Only one deserves mention here.

AURAMIN C. I. NO. 655

Synonyms: *Canary yellow. Pyoktaninum aureum. Pyoktanin yellow.*

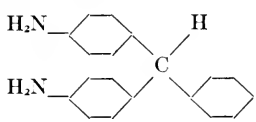


Altho of some use as a drug, auramin has little value in microscopic technic. It has been used by Fischel, however, in the vital staining of salamander larvae, and by Vinassa for staining plant sections.*

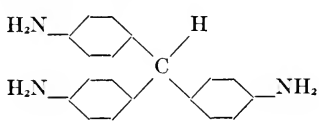
2. TRI-PHENYL METHANE DERIVATIVES

There are two groups of tri-phenyl methanes to concern us, the amino and the hydroxy derivatives. The former, which are much the more numerous, are very strongly basic, thanks to the amino groups, unless sulfonated like light green or acid fuchsin. The rosolic acid dyes, on the other hand, are hydroxy phenyl methanes, the amino groups being replaced by hydroxyl groups; they are therefore acid instead of basic dyes.

There are likewise two subdivisions of the amino derivatives, the di-amino tri-phenyl methanes and the tri-amino tri-phenyl methanes. These two groups are derivatives respectively of:
di-amino tri-phenyl methane



and tri-amino tri-phenyl methane, or pararosanilin.



The individual dyes of this series are substitution products of these

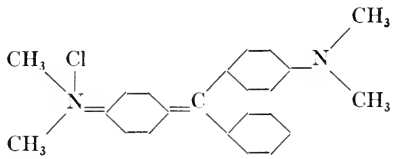
*Literature References to the procedures mentioned in this chapter may be found on pp. 110 to 128 and 138 to 145.

two compounds and differ from one another in the number of methyl, ethyl, or phenyl groups introduced, and according to whether they are introduced into the amino groups or directly onto the benzene rings.

a. Di-amino Tri-phenyl Methanes

MALACHITE GREEN C. I. NO. 657

Synonyms: *Emerald green. New victoria green. Diamond green. Solid green. Light green N.*



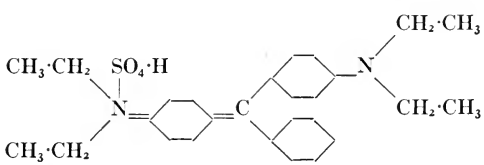
(*Absorption maxima: 616, [430]*)

Malachite green is a rather weakly basic dye that has been used in the past for various histological purposes; as by V. Beneden for staining Ascaris eggs, by Petroff for staining erythrocytes, and by Maas as a contrast stain following borax carmine. Today it has very largely been replaced by methyl green; but it is now often used by botanists for staining host tissue in plants infected with fungi, according to the technic of Pianese (with acid fuchsin and martius yellow), which was originally applied to cancer tissue.

BRILLIANT GREEN C. I. NO. 662

Synonyms: *Ethyl green. Malachite green G.*

This is a basic dye which is generally known in the form of the sulfate:

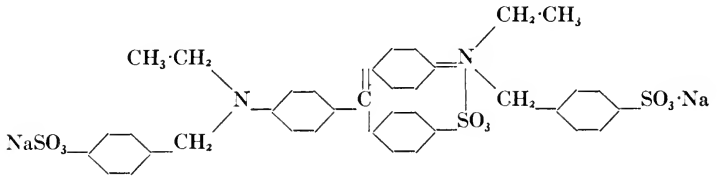


(*Absorption maximum: 623.*)

The largest call for brilliant green at present is as an indicator in media for water analysis, according to the technic of Krumwiede. It is also used for inhibiting the colon organism in stools; and is added to broth for the enrichment culture of the typhoid bacillus.

Synonyms: *Light green 2G, 3G, 4G,* or *2GN. Acid green* (with various shade designations). *Fast acid green N.*

This is a derivative of brilliant green, which is sulfonated and is therefore an acid dye.



(*Absorption maximum: 633.5.*)

Light green is a valuable plasma stain often used for staining tissues in contrast to iron haematoxylin, altho it fades badly if exposed to bright light. It is used by Benda in contrast to safranin as a cytoplasm stain for spermatozoa. In plant histology it is a useful cytoplasm and cellulose stain.

b. Tri-amino Tri-phenyl Methanes (Rosanilins).

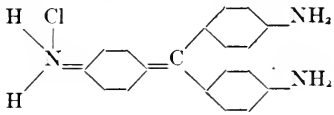
The simplest rosanilins are the dyes sold as basic fuchsin. This term seems to be somewhat loosely used to apply to two or three different dyes and to various mixtures of them. The dyes known as fuchsin differ from the methyl violets and other rosanilins in that the amino groups are not methylated or substituted in any other way. The fuchsins may, however, have methyl groups introduced directly onto the benzene rings instead of into the amino groups; and the different fuchsins vary from one another in the number of such methyl groups present. There are four primary compounds theoretically possible, namely with no methyl group, and with one, two, and three substituent methyl groups respectively. The types commonly encountered are listed below.

Basic fuchsin is a very valuable stain, and is one of the most powerful nuclear dyes. It is also a stain for mucin, for elastic tissue, and for bringing out the so-called fuchsinophile granules. It is often used for staining the nuclear elements of the central nervous tissue. It is one of the most useful bacterial stains, particularly in the Ziehl-Neelson method for differentiating the tubercle organism and thus diagnosing tuberculosis. As an indicator it is used to distinguish the typhoid organism from other closely related forms by means of the Endo medium, in which it is reduced to the colorless leuco-fuchsin by the use of sodium sulfite. This medium (which contains lactose) remains colorless in the presence of the typhoid organism, which does not attack lactose; but becomes colored in the presence of organisms like *Bacterium coli* which fer-

ment the lactose (a reaction that converts the leuco-fuchsin again into the dye fuchsin).

PARA-FUCHSIN C. I. NO. 676

Synonyms: *Basic rubin. Pararosanilin. Para-magenta.*

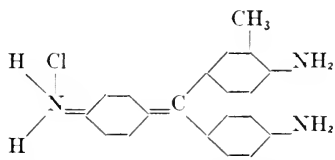


(*A basic dye; absorption maximum about 539.*)

This dye is frequently sold as basic fuchsin. Investigation, in fact, shows that most of the stains sold under that name are really pararosanilin chloride. The acetate is also sometimes encountered. It is evident that for many purposes for which fuchsin is used, this is satisfactory; but a recent investigation by the Commission shows that it is ordinarily less desirable than the higher homologs, especially rosanilin.

ROSANILIN

This compound is mono-methyl fuchsin, or triamino-tolyl-diphenyl-methane chloride.



(*A basic dye; absorption maximum about 542.*)

It is not a textile dye, and is not found free from pararosanilin unless specially prepared. One stain company, it is found, has supplied it as "basic fuchsin, for Endo medium." For this particular purpose, indeed, rosanilin hydrochloride proves to be the best adapted of any of the basic fuchsins; and, so far as it has been investigated, it is a very satisfactory stain for all purposes for which basic fuchsin is ordinarily used.

BASIC FUCHSIN C. I. NO. 677

Synonyms: *Diamond fuchsin. Magenta. Rubin. Anilin red.*

Various shades occur, denoted by different designations.

This dye, as furnished for textile purposes, is a mixture in about equal parts of pararosanilin and rosanilin.

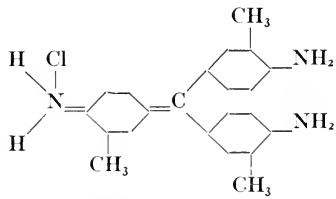
As the greater methyl substitution of the latter compound causes it to have a deeper shade than pararosanilin, the dye varies in

depth according to the amount of rosanilin present. These mixtures are the various fuchsins known to commerce. In the course of the recent investigation of fuchsins, no basic fuchsin of this type has been found offered to biologists as a stain. Whether it would prove satisfactory for staining purposes still remains to be determined.

NEW FUCHSIN C. I. NO. 678

Synonyms: *Isorubin. Fuchsin NB.*

This compound is tri-methyl fuchsin, or triamino tritolyl methane chloride:



(*A basic dye; absorption maximum about 544, 545.*)

This dye is sometimes sold for a stain under the name of basic fuchsin, altho the most reliable companies sell it under its correct name. Recent investigations show that it may be admirable for all the staining purposes for which basic fuchsin is used.

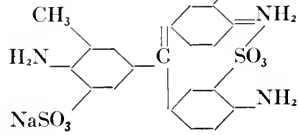
ACID FUCHSIN C. I. NO. 692

Synonyms: *Fuchsin S, SN, SS, ST,* or *S III. Acid magenta. Acid rubin.*

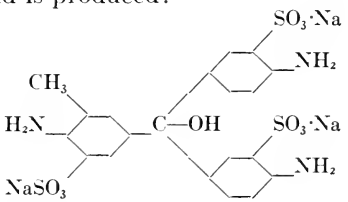
(*An acid dye; absorption maximum about 545.*)

This dye owes its acid character to the fact that it is a sulfonated derivative of basic fuchsin. Acid fuchsins are ordinarily rather complex mixtures. As there are four primary basic fuchsins possible, according to the degree of methyl substitution, and as each may yield at least three different compounds on sulfonation, fully a dozen acid fuchsins are theoretically possible, and samples are hardly to be expected which are not mixtures of several.

The generally accepted formula of one of the homologs present in acid fuchsin, namely the di-sodium salt of rosanilin trisulphonic acid, is:



The bond connecting one of the sulfonic groups with an amino group attached to a different benzene ring is assumed to exist in order to account for the fact that altho only two of the sulfonic groups are neutralized with sodium, the compound acts as tho it has no free acid. In other words, it is a case of intramolecular salt formation. Now when the tri-sodium salt is formed, this bond is broken down, whereupon the quinoid ring disappears and the following compound is produced:



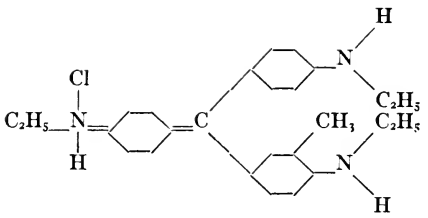
This compound, it will be seen, is a carbinol in structure, and as it lacks the quinoid ring it is colorless; but it is very readily converted into the di-sodium salt by the addition of acid, whereupon the color again appears. This property makes acid fuchsin of use as an indicator. The decolorized solution of acid fuchsin neutralized with sodium hydrate is called the Andrade indicator. It is used quite extensively in bacteriological work, because of the striking reaction when its color is restored by acid-forming bacteria. As an indicator to show hydrogen-ion concentration at all accurately, however, it is found to have much less value than the phthalein and sulphonphthalein dyes (see pp 83 to 86.)

Acid fuchsin is a widely used plasma stain, which has also been recommended for a number of special uses. Among the best known are: the Van Gieson connective tissue stain, in which it is used with picric acid after haematoxylin to differentiate smooth muscle from connective tissue; the Ehrlich-Biondi stain, in which with methyl green and orange G it is employed in histology and for staining blood smears; and the Ehrlich tri-acid stain for blood, which is a "neutral" combination with orange G and methyl green. In plant histology it is used to stain the cortex, pith and cellulose walls; while the Pianese stain (with malachite green and martius yellow), originally applied to cancer tissue, is now used by plant pathologists in studying infected vascular plants. It is used with methyl green, by Altmann, Bensley and Cowdry as a stain for mitochondria. To the pathologist it is quite valuable as a constituent (with anilin blue and orange G) of the Mallory connective tissue stain.

HOFFMAN VIOLET C. I. NO. 679.

Synonyms: *Dahlia. Iodine violet. Red violet. Violet R, RR, or 4RN.*

Hoffman violet is derived from fuchsin by the introduction of methyl or ethyl groups (generally the latter) into the amino groups. Thus the tri-ethyl rosanilin has the formula:



(*A basic dye.*)

The dyes known to commerce are mixtures of the higher and lower homologs of this series. The higher homologs, on account of the presence of ethyl groups and the higher molecular weight resulting therefrom are a very deep violet.

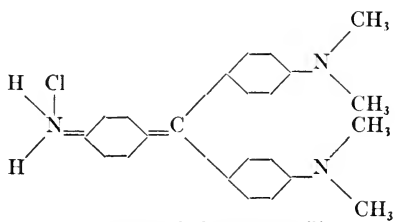
Hoffman violet has been used by Ehrlich and by Unna for staining mast cells; and by Juergens for staining amyloid, which stains red, while the cytoplasm is colored blue.

METHYL VIOLET C. I. NO. 680

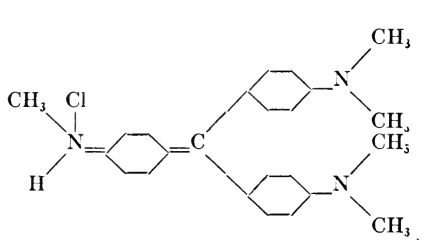
Synonyms: *Dahlia B. Paris violet, Pyoktanin blue. Gentian violet.*

Various shades denoted: Methyl violet 3R, 2R, R, B, 2B, 3B, BBN, BO, 3V.

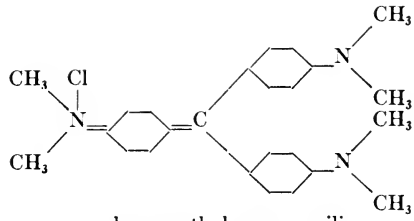
The various dyes denoted methyl violet are mixtures of tetra-, penta-, and hexa-methyl pararosanilin:



tetra-methyl pararosanilin



penta-methyl pararosanilin



hexa-methyl pararosanilin
(crystal violet)

(*Basic dyes; absorption maxima: 583-584 in 90% alcohol.*)

In the case of these compounds, as in the case of other series of homologs differing in extent of methylation, the shade is deepened by the introduction of each methyl group. Hence the various mixtures known to the trade as methyl violet vary from reddish to bluish violets according to the relative amounts of the more and less completely methylated compounds present in the mixture. This is the significance of the various shade designations listed above, R's indicating the reddish shades, and B's the bluish shades. Of these various shades the bluer ones seem to be best for biological purposes, methyl violet 2B having been found satisfactory for practically all purposes for which methyl or gentian violet is ordinarily called for. This indicates that the biologist requires the higher homologs in this group. Now the most completely methylated methyl violet is the hexa-methyl compound, which is easily obtained pure and is known to the trade as crystal violet. This dye, therefore, appeared very interesting to the Commission and has been given considerable investigation.

CRYSTAL VIOLET C. I. NO. 681

Synonyms: *Violet C, G,* or *7B. Hexamethyl violet. Methyl violet 10B. Gentian violet.*

(*A basic dye; absorption maximum about 591.*)

This dye is hexa-methyl-pararosanilin, whose formula is given above as one of the components of methyl violet.

The Commission has made as careful an investigation of this dye as of any other and has become very enthusiastic over it. Methyl or gentian violet is of chief value to the biologist as a nuclear or chromatin stain, having many histological and cytological applications, the one for which it is most commonly used at present being the Flemming triple stain in which it is employed with orange G and safranin—a technic which gives a very high degree of differentiation. It is also used for staining amyloid in frozen sections of fresh and fixed tissue, and for staining the platelets in blood; while it is much used by the Weigert technic for stain-

ing fibrin and neuroglia. The bacteriologist also finds it a useful stain and probably purchases more at the present time than all other biologists together; the chief bacteriological use is in the Gram technic for distinguishing between different kinds of bacteria. A further more recent use is in bacteriological media for inhibiting the growth of Gram-positive organisms, due to its selective bacteriostatic action.

The Flemming and Gram stains have seemed the most delicate procedures for which it is used; so they have been given the most careful study. In the case of the Gram stain it was discovered that there are a score or more different procedures all referred to by the name "Gram" stain, and a study was made of all the methods that were found (see Hucker and Conn 1923). The result of the investigation is to conclude without reservation that crystal violet may be substituted for gentian violet in both the Gram and Flemming technics, and probably for gentian or methyl violet in any of the bacteriological or histological methods for which either stain is designated. If crystal violet can be used in all cases, the advantage is obvious; for it is a definite chemical compound, while methyl and gentian violet are both variable mixtures.

It is of interest to note that in the literature of microscopic technic crystal violet has been specified instead of gentian violet for some special procedures. Worth noting is Benda's crystal-violet-alazirin method for staining chondriosomes, and its modifications by Meves and Duesberg; and also its use in combination with erythrosin by botanists for staining lightly lignified walls, in which technic it proves more uniform than gentian violet.

GENTIAN VIOLET

A poorly defined mixture of violet rosanilins is well-known to biologists under the name gentian violet. The name is not used at present in the dye or textile industries, however, and for this reason the dye is not listed in dye indexes. It apparently applies to a certain mixture containing about half dextrin and half dye, the dye being a methyl violet, that is a mixture of crystal violet with lower homologs of the same series. The statement has been made and often repeated in biological literature that gentian violet is a mixture of crystal and methyl violet; but the looseness of the statement is evident when it is realized that crystal violet is a component of all the deeper shades of methyl violet. It is possible that before the war gentian violet did represent a fairly constant mixture, but there seems to be some doubt even on this point. It is certain that since the war each company has used its own judgment as to what to furnish when gentian violet is ordered. One company admits furnishing crystal violet unmodified under this name; another claims to be supplying penta-methyl-pararosanilin for gentian violet; another apparently has been mixing crystal violet and

methyl violet 2B together with an equal weight of dextrin. Yet in actual test, none of these preparations proves any better than crystal violet or methyl violet 2B, unless it be possibly in inhibiting the growth of Gram-positive bacteria. Unfavorable reports of crystal violet for this purpose have been received; but tests of several samples of crystal violet now on the market show them to be bacteriostatic to a higher degree than the sample of pre-war gentian violet with which they were compared.

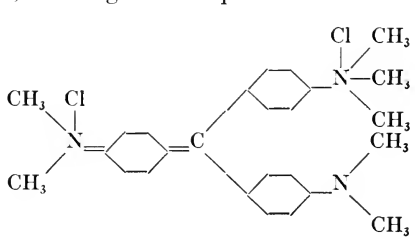
Under the circumstances the Commission has faced a difficult problem in trying to standardize gentian violet. The question has been whether to recognize the name at all, or to approve some particular dye or mixture of dyes of this group as gentian violet. The former course was almost impossible because of the wide demand among biologists for gentian violet under that name. The second course (unless considerable latitude be recognized) would be entirely arbitrary, inasmuch as no information is available to show which members of this group of dyes are especially needed in histology or bacteriology. Accordingly the Commission has finally defined gentian violet as either penta-methyl or hexa-methyl pararosanilin, or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B. Gentian violet, must, moreover, prove satisfactory in actual use as determined by the tests proposed by the Commission (see specifications page 135). This definition will not exclude anything now sold as gentian violet except those that do not prove satisfactory in performance. At the same time the Commission does not wish to give official standing to gentian violet and recommends that crystal violet be ordinarily ordered in its place.

METHYL GREEN C. I. NO. 684

Synonyms: *Double green. Light green.*

(A basic dye; absorption maximum about 633.8)

Methyl green is crystal violet into which a seventh methyl group has been introduced by the action of methyl chloride or methyl iodide upon it, forming the compound:*



*This ordinarily occurs in trade as a zinc double salt.

As the seventh methyl group is very loosely attached, there is always some methyl violet present, either because it is not all completely converted into the higher homolog or because it has broken down again. It has been stated that to obtain free methyl green the commercial dye should be shaken in a separatory funnel with amyl alcohol or chloroform, which dissolves the methyl violet. As a matter of fact, however, pure methyl green may not be always desired by the biologist, as the dye owes part of the metachromatic properties for which it is prized to the presence of small amounts of the violet compound.

Methyl green is at present one of the most valuable nuclear stains known to the histologist, and is widely used as a chromatin stain by the cytologist. On the other hand it has been used by Galeotti as a cytoplasm stain following acid fuchsin and picric acid. In the Ehrlich-Biondi technic it is used to stain nuclei in contrast to acid fuchsin; while Bensley employs it to stain chromatin in contrast to acid fuchsin which stains the mitochondria. It is an ingredient of the Ehrlich triacid mixture (with orange G and acid fuchsin) for staining blood smears. Botanists find it a valuable stain, combined with acid fuchsin, for lignified xylem. One of its most valuable uses today is in the Pappenheim stain, in which it is combined with pyronin and used for staining the gonococcus and mast cells as well as by Unna in studying chromolysis. It is also a useful chromatin stain for protozoa, and is employed in weak acetic acid solution for staining fresh material beneath the coverglass.

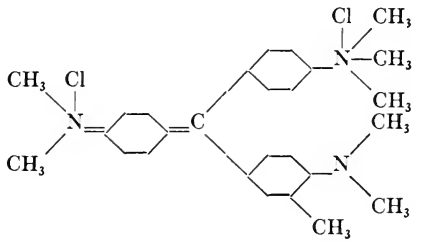
When the foreign supply of dyes was first shut off, this stain proved one of the most difficult to obtain in satisfactory quality, largely due to the looseness with which the seventh methyl group is attached and the resulting instability of the compound. At first certain green dyes of an entirely different nature were furnished, but as soon as an investigation of the dye was begun manufacturers proved perfectly able to produce methyl green; the difficulty came in obtaining the right degree of purity. Samples were finally furnished so pure that they lacked completely the necessary metachromatic staining quality; and it proved necessary to add a certain small percentage of the violet dye to obtain the proper results. This problem seems to have been solved at present and satisfactory methyl green is available. The chief problem now is to standardize it. With other stains this can ordinarily be done on the batch basis, approving some batch large enough to meet the demand for a period of years. With methyl green this cannot safely be done, on account of its instability. Hence large batches are impractical; and the stain ought to be sold with the caution that the dye does not keep indefinitely without change. That this is not generally realized is shown by the fact that when a certain company recently announced for sale a supply of German stains imported before the war and kept on their shelves since then, one laboratory ordered a

pound of methyl green. For the purposes for which this stain was desired this batch may be satisfactory; but the user can hardly count on its being the same as it was when imported nor on its remaining unchanged until used up.

IODINE GREEN C. I. NO. 686

Synonym: *Hoffmann's green.*

This dye is closely related to methyl green, the generally accepted formula being:



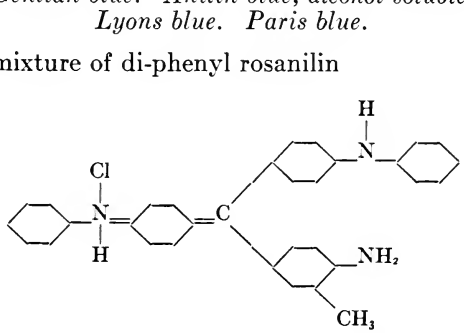
(*A basic dye.*)

Iodine green is a nuclear or chromatin stain which has selective properties that make it of value in certain special procedures. It is used by Ciaccio for nervous tissue in combination with acid fuchsin and picric acid; and by Lefas as a blood stain in combination with acid fuchsin. It is used by Zimmermann with basic fuchsin for staining chromatin in plant tissue; while together with acid fuchsin it is occasionally used by botanists for staining lignified xylem. It is also used for staining mucin and amyloid, having the property of giving the latter a red instead of a green color.

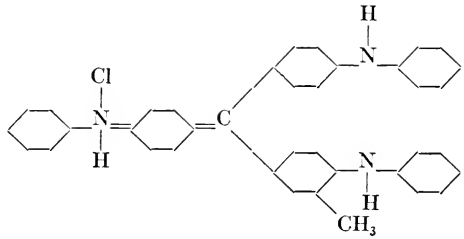
SPIRIT BLUE C. I. NO. 689

Synonyms: *Gentian blue. Anilin blue, alcohol soluble. Night blue. Lyons blue. Paris blue.*

This is a mixture of di-phenyl rosanilin



and tri-phenyl rosanilin:

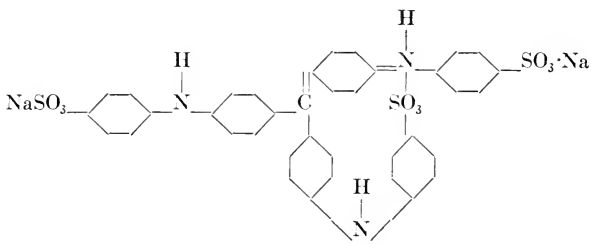


(*Basic dyes; absorption maximum of spirit blue 2R about 581 in alcohol.*)

It is used in contrast to carmin in staining embryonic tissues; it brings out growing nerve fibers well.

METHYL BLUE C. I. NO. 706

Synonyms: *Cotton blue. Helvetia blue.*



(*An acid dye; absorption maximum about 607.*)

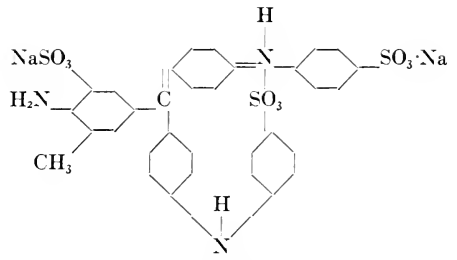
On account of the sulphonic groups, this dye is strongly acidic and makes a good counter-stain. It is used by Mann with eosin for staining nerve cells; and by Dubreuil, combined with picric acid, in contrast to a red nuclear stain such as carmin or safranin.

ANILIN BLUE, W. S. (i.e., water soluble) C. I. NO. 707

Synonyms: *China blue. Soluble blue 3M* or *2R. Marine blue. Cotton blue. Water blue. Berlin blue.*

This dye is a mixture of the tri-sulphonates* of tri-phenyl para-rosanilin (C. I. 706) and of di-phenyl rosanilin. The latter is:

*The location of the sulphonic groups is uncertain.



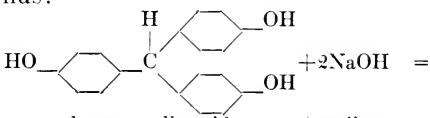
(An acid dye; absorption maximum of water blue 2B about 546.5.)

It is a widely used histological stain, having valuable counterstaining properties. It is also of use as an indicator, due to the disappearance of the color upon complete neutralization, as in the case of acid fuchsin. As an indicator, however, it has the disadvantage that the blue color is but slowly restored upon addition of acid.

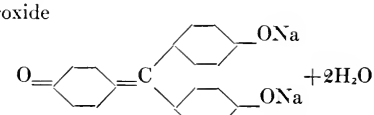
Its chief histologica uses are: by Stroebe and Huber as a cytoplasm stain preceding safranin; by Galli for axis cylinders; frequently by botanists as a contrast for safranin in vascular plant tissue, or for magdala red in algae; and very widely by pathologists in the Mallory connective tissue stain, in which it is combined with orange G and acid fuchsin; by Unna in contrast to orcein for staining epithelial sections, and in studying the process of chromolysis.

(c) Hydroxy Phenyl Methanes (Rosolic Acids)

The rosolic acid dyes, as stated above, are tri-phenyl methane derivatives in which the amino groups of the rosanilins are replaced with hydroxyl groups, thus giving them acidic instead of basic character. The compounds of this group are not very important as dyes and are scarcely used as stains. The greatest interest of the biologist in them is due to their use as indicators, since in acid solution the quinoid ring disappears and the compound becomes colorless, while alkali changes it back to the colored form. Thus:

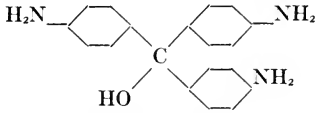


leuco-rosolic acid + sodium hydroxide =

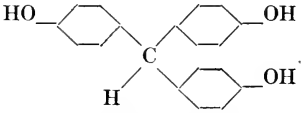


disodium rosolate + water

There is considerable confusion in the nomenclature of these dyes, as the names employed may be used in a strict chemical sense or in a looser sense in practice. Chemically there are two rosolic acids, which are related just as are rosanilin and pararosanilin. Pararosolic acid differs from pararosanilin, only in having hydroxyl groups in place of the amino groups:

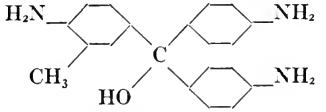


Pararosanilin carbinol

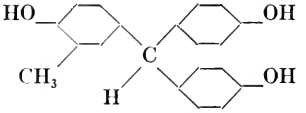


leuco-pararosolic acid

Rosolic acid, on the other hand, is a mono-methyl derivative, and bears the same relation to rosanilin:



Rosanilin carbinol



leuco-rosolic acid

Now the dye to which the name rosolic acid or aurin is generally given in practice is a mixture consisting of both rosolic acid and pararosolic acid together with other closely related compounds. This dye is:

AURIN OR ROSOLIC ACID C. I. NO. 724

A mixture of rosolic acid and pararosolic acid, with oxidized and methylated derivatives of the latter. This product is of considerable use as an indicator.

Corallin yellow is the name given to its sodium salt.

No other dyes of this group have biological use. Two others perhaps deserve mention:

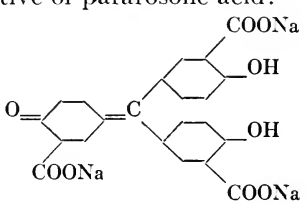
CORALLIN RED C. I. NO. 726

Synonym: *Aurin R.*

Apparently a compound dye, the pararosanilin salt of pararosolic acid.

CHROM VIOLET C. I. NO. 727

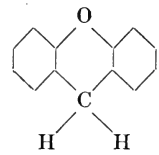
A carboxyl derivative of pararosolic acid:



CHAPTER VII

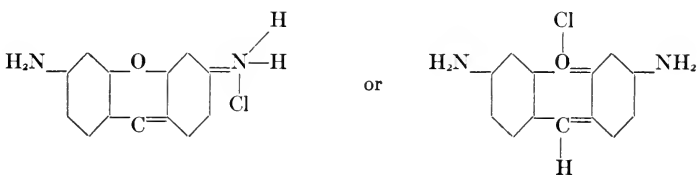
THE XANTHENE DYES

THE group of colored compounds known as xanthese dyes comprises a number of basic and acid dyes and quite a series of indicators. In fact, the most valuable indicators known to the chemist fall in this group. They are derivatives of the compound xanthene:

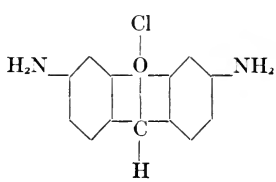


1. THE PYRONINS

The pyronins are methylated di-amino derivatives of xanthene. They are closely related to the diphenyl methanes and are sometimes classed with them, as they have a carbon atom attached to two benzene rings, and show the same tendency toward quinone structure. Their formula, on the other hand is like that of the oxazins except that the nitrogen of the central ring is replaced by a methenyl (CH) radical. Like the oxazins, the atomic grouping may be assumed to be in either the paraquinoid or the ortho-quinoid form, thus:



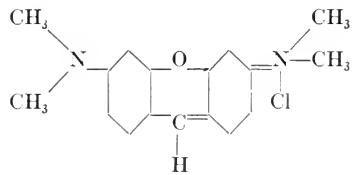
Another arrangement of the atoms is possible in which no quinoid ring exists, namely:



This latter form might also be assumed for the oxazins and thiazins as well, and this type or formula is frequently used for the azins; but the xanthene dyes are more often represented in this form. If this formula is adopted the quinoid ring cannot be accepted as

their chromophore. For this reason one of the quinoid formulae seems preferable; and for the sake of uniformity the paraquinoid form will be given in the following pages. It must be remembered, however, that the other formulae are equally admissable; and it is possible that the compound occurs in two or even all three of the different forms.

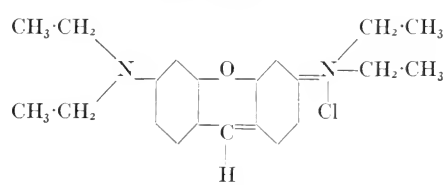
PYRONIN G C. I. NO. 739



(*A basic dye; absorption maximum about 545.*)

This dye finds its principal use as a stain in the Pappenheim combination, where it is employed with methyl green for staining basophile elements, especially the mast cells, and for staining the gonococcus in smears of pus. It is also used sometimes as a counter stain in the Gram technic for bacteria; and by Ehrlich as a component of certain "neutral" stains. Since the war it has proved difficult to obtain this dye in America except by importation, as the intermediate from which it is manufactured is very difficult to prepare and is not produced in this country.*

PYRONIN B C. I. NO. 741



(*A basic dye; absorption maximum about 550.*)

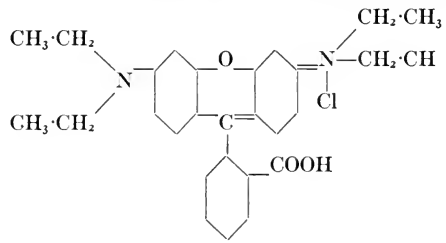
This dye differs from pyronin G only in that it is an ethyl instead of a methyl derivative. As a result it is very slightly deeper in shade but has almost identically the same staining behavior. Investigations recently carried on by the Commission indicate that it can replace pyronin G in the Pappenheim stain and probably in all its other uses. This is very encouraging, for it is much more easily prepared and a very satisfactory product of American manufacture is now available.

*For literature references to the procedures mentioned in this chapter see pp 110 to 128 and 138 to 145.

The rhodamines are similar to the pyronins except that there is a third benzene ring attached to the central carbon atom and attached to this ring is a carboxyl group in the ortho position. This latter group, altho of acid tendency, does not counteract the basic action of the amino groups, so the dyes are basic in character. Only one of them is of any significance to the biologist, namely:

RHODAMINE B C. I. NO. 749

Synonyms: *Rhodamine O. Brilliant pink.*

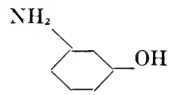


(*A basic dye; absorption maxima about 556.5, [519]*)

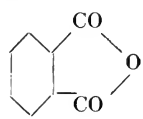
A rhodamine, probably the above dye, has been used by Griesbach with osmic acid to fix and stain blood simultaneously; by Ehrlich as a component of "neutral" stain mixtures; by Rosen for histological work in contrast to methylene blue; and by others in contrast to methyl green.

A somewhat different dye, known as Rhodamine S (C. I. No. 743) has been mentioned in the same connection and may have been used for some of the above mentioned purposes. It is not a true rhodamine, however, but belongs to a closely related group of compounds, the succineins; for it does not have the three benzene rings, the radical C_6H_4·COOH being replaced by C_2H_4·COOH.

In practice the rhodamines are prepared not from xanthene but by the condensation of two molecules of meta-amino phenol,



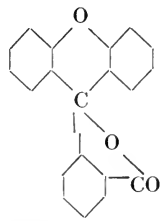
with one of phthalic anhydride:



This shows their close relation to the next group of dyes, namely the fluorane derivatives, which as will be seen are also prepared from phthalic anhydride. In fact these two groups of dyes, acid and basic respectively, are related in exactly the same way as the rosolic acids and the rosanilins, the one group having hydroxyl radicals where the other has amino groups.

3. FLUORANE DERIVATIVES

Fluorane is not a dye, but is a very important compound in dye chemistry. It is a derivative of phthalic anhydride, and contains a xanthene ring (five C atoms and one O atom) as well as a lactone ring (four C atoms and one O atom) besides three benzene rings; thus:



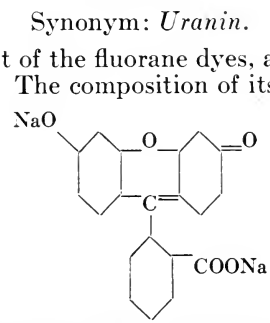
The fluorane dyes are derivatives of this by the introduction of hydroxyl groups into two of the benzene rings at the para position to the central carbon atom and the further introduction of halogen atoms at various positions in all three benzene rings.

It proves convenient here to class these compounds with the xanthene dyes. They may, however, be equally well considered tri-phenyl-methane dyes, as can be seen by a glance at the formula of any of them; in fact they are generally so considered by the chemists. To the biologist they stand in a distinctly different class from the tri-phenyl-methanes; and for that reason are treated here instead of in the preceding chapter.

FLUORESCEIN C. I. NO. 766

Synonym: *Uranin.*

This is the simplest of the fluorane dyes, and is the mother substance of the eosins. The composition of its sodium salt is:



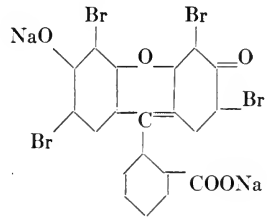
(An acid dye; absorption maximum about 490.)

Synonyms: *Water soluble eosin. Eosin W* or *WS.*

Various shades denoted: Eosin G, Y extra, S extra, J extra, B extra, GGF, 3J, 4J, KS, DH and JJF.

(*An acid dye; absorption maximum about 516.*)

This dye is typically tetrabrom fluorescein:



but the mono- and di-brom derivatives are also known and frequently occur in eosin. This affects the shade, as the more bromine present the redder the dye. It is plain that various mixtures of these compounds are on the market; but it has not yet been determined which are more suitable for biological purposes. Considerably more work on eosin is needed than has been done at the present time. From the name "water soluble eosin" it is often assumed that this dye is not soluble in alcohol. This is not true, however.

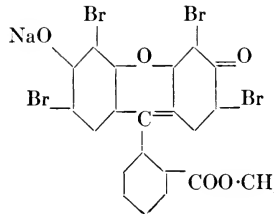
Yellowish eosin is one of the most valuable plasma stains known. It is used in various technics for staining the oxyphile granules of cells (i.e., the granules having special affinity for acid dyes); these cell elements, in fact, being often called eosinophile granules because their presence was first recognized thru the use of this dye. It is often employed as a counterstain for haematoxylin and the green or blue basic dyes; as for example by Mallory with methylene blue,* and by List with methyl green. It is used by Mann mixed with methylene blue as a tissue stain; and by Teichmuller for staining sputum before staining with methylene blue. At the present time one of the uses for which it is in greatest demand is as a blood stain in the technic of Romanovsky, with its various modifications, in which it is combined with methylene blue to form a "neutral" stain.

METHYL EOSIN C. I. NO. 769

Synonym: *Eosin, alcohol soluble.*

This is the methyl ester of yellowish eosin, the sodium salt of which is:

*See, however, the statement about this technic below under phloxine (p. 82).

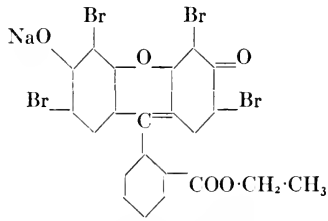


(*An acid dye; absorption maxima about 520, [485.5]*)

ETHYL EOSIN C. I. NO. 770

Synonyms: *Eosin, alcohol soluble. Eosin S.*

This is similar to methyl eosin, but is the ethyl ester:



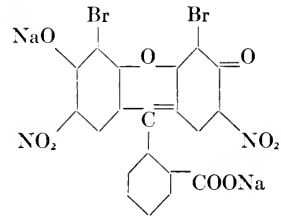
(*An acid dye; absorption maxima about 523.5, [487]*)

An alcohol soluble eosin, either this dye or the preceding, is a valuable counterstain after Delafield's haematoxylin in general animal histology.

EOSIN, BLUISH C. I. NO. 771

Synonyms: *Eosin BN, B, BW,* or *DKV. Safrosin, Eosin scarlet B* or *BB. Scarlet J, JJ, V. Nopalin G. Caesar red.*

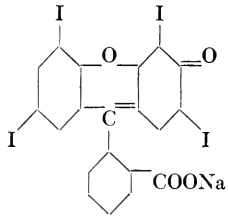
This is a dibrom derivative of dinitro-fluorescein.



(*An acid dye; absorption maxima about 521.5, [486]*)

Synonyms: *Erythrosin R* or *G. Pyrosin J. Dianthine G. Iodo-eosin G.*

This is a fluorescein in which there are two substituent iodine atoms instead of four bromine atoms as in yellowish eosin.

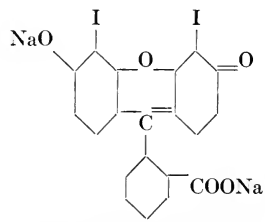


(*An acid dye; absorption maximum about 510.5.*)

ERYTHROSIN, BLUISH C. I. NO. 773

Synonyms: *Erythrosin B. Pyrosin B. Eosin J. Iodo-eosin B. Dianthin B.*

This is the tetraiodo compound corresponding to the tetrabrom compound of typical eosin.



(*An acid dye; absorption maximum about 524.*)

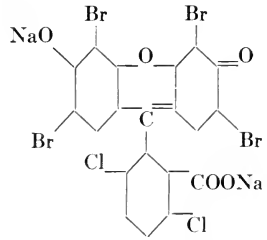
Erythrosin has some use as an indicator. It is also employed as a contrast stain for haematoxylin and certain blue and violet nuclear stains. Held uses it, preceding methylene blue, as a plasma stain for nerve cells. It is employed by Winogradsky for staining bacteria in soil. For these purposes probably the tetraiodo compound (i.e., erythrosin bluish) is desired; but the literature is vague on the subject.

A sample of erythrosin of pre-war origin that was labeled Magdala red has been examined by the Commission. This mislabeling undoubtedly explains Chamberlain's results already mentioned (page 57) in staining algae. Chamberlain, it will be recalled, was able to obtain good results with a low priced product called Magdala red but not with the high priced stain called Magdala red *echt.*

Synonyms: *Erythrosin BB. New pink.*

(An acid dye; absorption maximum of C. I. No. 778 about 537.5.)

If fluorescein is prepared from dichlor or tetrachlor-phthalic acid, instead of from simple phthalic acid, its derivatives have a deeper, more pleasing shade than the ordinary eosins. Of these derivatives, phloxine contains four bromine atoms and thus corresponds to Eosin Y. It contains two or four chlorine atoms according to whether it is prepared from the dichlor- or the tetrachlor-phthalic acid; and there are two Colour Index numbers corresponding to these two compounds, the former being No. 774, the latter No. 778. It is uncertain which is desired by the biologist. The dichlor compound is:



Unna uses phloxine in combination with several other acid dyes in studying the process of chromolysis. The dye has seldom been specified for biological work; yet there is reason to believe that it is a more valuable stain than anyone has realized in the past, and that it has frequently been used under other names.

Chamberlain (1924 page 58) mentions having used it successfully in place of Magdala red in staining algae. His original technic called for Magdala red; but true Magdala red does not serve his purposes. Inasmuch as erythrosin (see above) was evidently sold in the past as Magdala red and Chamberlain can duplicate his original results with phloxine, the chances are that some of the Magdala red formerly available was either phloxine or else that phloxine and erythrosin give similar results by Dr. Chamberlain's technic.

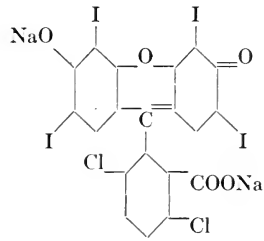
It has, furthermore, been found that no sample of eosin at present available of either domestic or foreign origin works in the well-known Mallory eosin-methylene-blue stain; but after investigating both phloxine and rose bengal, Dr. Mallory reports the former to be "the best *eosin* I have yet found for use in the eosin-methylene blue stain for parafin sections of tissues fixed in Zenker's fluid."* Here again is a case where phloxine apparently was obtained before the war under an incorrect name and the incorrect name used in the publication of a well-known technic.

*Quoted from personal letter.

Various shades denoted: Rose bengal B, 2B, 3B.

(*An acid dye; absorption maximum of C. I. No. 779 about 548.*)

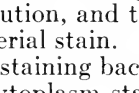
This is a second derivative of di-chlor or tetra-chlor fluorescein, and corresponds to erythrosin, as it contains four iodine atoms. As in the case of phloxine, there are two similar dyes, the di-chlor compound (No. 777) and the tetra-chlor compound (No. 779); it is not certain which is desired in microscopic work. The former has the formula



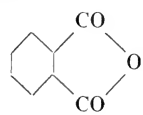
This dye has a pleasing deep pink color; and altho an acid dye it proves to have considerable affinity for bacterial protoplasm, if used in carbolic acid solution, and to have good selective properties when used as a bacterial stain. It has recently been recommended by the author for staining bacteria in soil suspensions. It has also been used as a cytoplasm stain following haematoxylin.

4. PHENOLPHTHALEIN AND THE SULPHONPHTHALEINS

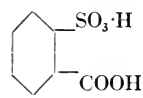
A phthalein is a compound of phthalic acid:



or rather of phthalic anhydride:

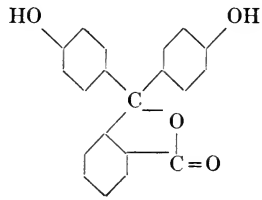


with phenol or a phenol derivative. If phthalic acid is heated with phenol and sulfuric acid it combines with two molecules of the latter and forms phenolphthalein. In the same way, a sulphonphthalein is a compound of ortho-sulpho-benzoic acid:

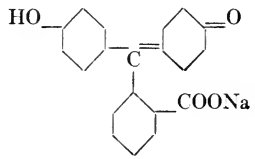


and phenol or a phenol derivative. These compounds, altho sometimes behaving as dyes, are not used as dyes or stains, but as indicators. For this purpose the members of the group are very valuable.

Phenolphthalein, altho not used as a dye, is colored and is apparently capable of salt formation. In acid solutions it is colorless, and is assumed to have the formula:



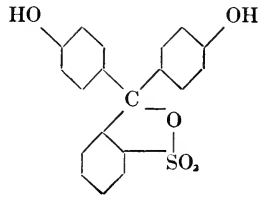
Upon neutralization the alkali is believed to attach itself to the CO-group, which breaks the five-sided ring (the lactone ring) and causes one of the benzene rings to take on quinoid form, thus:



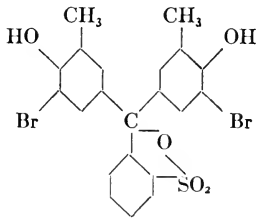
With this change, the red color of the compound appears, but disappears again if the solution is made acid so as to destroy the quinoid structure. This makes the compound a very valuable indicator.

The sulphonphthaleins are not commercial dyes, altho capable of acting as acid dyes. Their real value is as indicators. Quite a long series of them has been prepared, which in general show their deepest color in alkaline solutions and turn yellow on the addition of acid. Fortunately nearly every one of them has a different point in the hydrogen-ion scale at which its color change begins, so that between them they indicate very accurately the hydrogen-ion concentration of solutions of any reaction ordinarily encountered. Some of them, such as thymol-sulphonphthalein (thymol blue), show two colors besides yellow, one in strong acid solutions and the other in strong basic solutions, while in solutions near the neutral point they are yellow. That these color changes are due to alterations in the structure of the molecule, such as the disappearance and reappearance of the quinoid ring, is generally assumed; but in the case of these compounds the relation of structure to color is complicated and has not yet been worked out to general satisfaction.

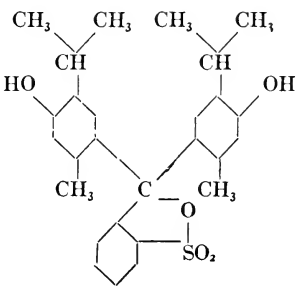
Phenol-sulphonphthalein (*phenol red*) has the formula:



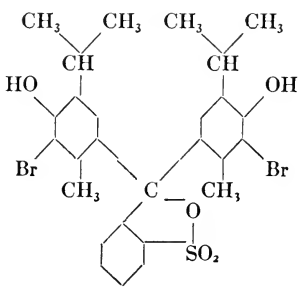
Among the other important sulphonphthalein indicators: *Brom cresol purple* is di-brom-ortho-cresol-sulphonphthalein:



Thymol blue and *Bromthymol blue* are thymol-sulphonphthalein and di-brom-thymol-sulphonphthalein respectively; thus:



and



The formulae are all so similar that there is no need of giving the others. It is enough to state that *brom phenol blue* is tetra-brom-phenol-sulphonphthalein; *cresol red* is ortho-cresol-sulphonphthalein; *brom cresol green* is tetra-brom-meta-cresol-sulphonphthalein; *chlor phenol red* is di-chlor-phenol-sulphonphthalein; *brom phenol red* is di-brom-phenol-sulphonphthalein; while *brom-chlor phenol blue* is di-brom-di-chlor-phenol-sulphonphthalein.

CHAPTER VIII

COMPOUND DYES

THERE are two ways in which dyes may be compounded. In the first place it is possible to mix mechanically any two dyes, and if they are of different colors with different selective powers, double staining effects may be procured. In the second place, it is often possible to form a chemical union between two dyes and thus to obtain an entirely new compound which may have quite striking staining properties. It is such compounds as these, rather than simple mechanical mixtures, that are ordinarily referred to as compound dyes.

The simple anilin dyes, it will be recalled (see Chapter II), owe their properties as dyes or as biological stains to the basic or acidic character of the dye molecule. Those parts of the protoplasm which are acid in nature (e.g., chromatin) tend to react with the basic dyes and to be colored with them; while those which are basic (e.g., cytoplasm) react similarly with the acid dyes. (This, to be sure, is not the whole theory of staining, as the process is quite complex and involves physical and mechanical factors as well; but it serves to illustrate the difference between the two kinds of stains.) Now, as already explained, the dyes are not used as free acids or free bases; but rather as sodium or potassium salts of the acid dyes, and as chlorides (or salts of some other colorless acid) of the basic dyes.

It is well known that when two salts, such as sodium chloride and ammonium nitrate, are mixed in solution there is an interchange of ions, and the resulting solution, when it reaches equilibrium, contains not only the original salts but also the four free ions and the two alternate compounds as well, in this case sodium nitrate and ammonium chloride. Now if one of these two new compounds happens to be insoluble, as silver chloride for example, which would have been formed if silver nitrate had been substituted for ammonium nitrate, it is thrown out of solution and equilibrium is not reached until the solution is free (or at least practically free) from the two ions which are insoluble in combination. In the same way, when a sodium salt of an acid dye and a chloride of a basic dye are mixed in solution, there is a similar tendency for the ions to interchange. Ordinarily the dyes are weaker acids and bases than the chlorine and sodium ions respectively; and if the compound dye formed were soluble in water there would be little chance for much of it to be produced. As a matter of fact, however, it is generally insoluble and is therefore thrown out of solution; hence the compound dye can be formed in considerable quantity.

Now compound dyes of this sort are sometimes referred to as neutral dyes or neutral stains. This terminology, of course, does

not indicate that they are neutral in reaction any more than do the corresponding terms acid and basic dyes. A dye chemist, in fact, uses the term neutral dye in an entirely different sense; but it is frequently employed by biologists, especially by Ehrlich, to refer to the basic dye salts of dye acids. In this chapter these compound dyes will be called *neutral stains*, as this latter term is not employed by dyers or dye chemists in any sense, and is unlikely, therefore, to be misunderstood. Erhlich also uses another term to apply to some of these compounds, namely "tri-acid dyes." He uses this term on the assumption that, while in the ordinary basic dyes only one of the three affinities of the dye for acid is satisfied, it is possible to satisfy all three and in this way to saturate the basic dye with acid. This assumption of his seems to be incorrect; but Ehrlich's "tri-acid stain" (see below) is so well-known that an explanation of the term is necessary.

It is possible to obtain endless variety of such dyes; but in practice only a certain number of them have proved useful. Among the basic dyes the most suitable for this purpose are methylene blue and the rosanilins (which act as strong ammonium bases); among the acid dyes eosin and the sulphonic acids (e.g., orange G and acid fuchsin).

Altho the neutral stains are insoluble in water, they are soluble to a greater or less extent in excess of either the acid or the basic dye. Thus if a watery solution of acid fuchsin is neutralized by adding drop by drop a watery solution of methyl green, there is at first no precipitation, because the methyl green salt of acid fuchsin is kept in solution by the excess of acid fuchsin. After the proper amount of methyl green has been added, however, and the mixture has stood long enough for the reaction to take place, the neutral dye is precipitated and the solution becomes nearly colorless. Then if more methyl green is added the neutral dye is slowly dissolved again; but as a rule neutral dyes are less soluble in excess of base than in excess of acid.

As simple aqueous solutions of these dyes are impossible and as alcoholic solutions of dyes do not stain well, various methods are employed to secure their action on the tissues. In some instances they are kept dissolved by the presence of an excess of acid or base (particularly the former); in others a certain quantity of acetone or methylal is used to hold the neutral dye in solution; sometimes (as in the original Romanovsky stain) the compound dye is used immediately after mixing, before the reaction is complete or precipitation has taken place; or again (as in the Wright stain) methyl or ethyl alcohol may be used as a solvent, and then after applying the alcoholic solution to the slide it may be diluted with water. This latter method is particularly efficacious, because the dissociation which takes place upon the addition of water causes the production of various dye compounds which may stain intensively and very selectively.

It is assumed that these compound dyes act on the protoplasm somewhat as follows: Certain parts of the cell have an affinity for the neutral stain and take it up as such; others, having an affinity for the basic dye, break up the neutral stain so as to obtain the basic portion of it, or if dissociation has taken place, take up the basic ion directly; while other parts of the cell with an affinity for acid dyes similarly combine with the acid portion of the stain. These three types of cell structures are known as neutrophile, basophile and oxyphile elements, respectively. The differentiation thus produced gives the neutral dyes their great value.

Ehrlich's "Triacid Stain."

The first neutral stain proposed for microscopic work was the "triacid stain" (see Ehrlich 1910, I, 227). In forming this compound dye, acid fuchsin and orange G are mixed in solution and to the mixture is then added such a quantity of methyl green that there is still an excess of the acid dye. This excess of the acid dye allows the neutral stain to stay in solution. The dye thus formed is a very valuable blood stain, and brings out finely the different structures in the leucocytes.

Slight modifications of this triacid stain have been used for tissues. The best known of these modifications is that of Biondi-Heidenhain.

Eosin-Methylene-blue Compounds*

The first worker to combine eosin and methylene blue was Romanovsky (1891). He realized that a mixture of these two dyes had great selective properties as a stain, and showed it to be excellent for blood, particularly in bringing out the malarial parasite. He also appreciated that it was more than a mere mixture of the two dyes and that some new dye having the property of giving the nuclei a red color was present. It was some time later before the nature of this new dye was known, altho it was subsequently named azure I or methylene azure; its true chemistry has scarcely been understood until very recently (see p. 48). Methylene violet, which probably was also present, had already been described by Bernthsen (1885). How these new dyes were formed in the Romanovsky stain was not known then; altho Romanovsky stated that different lots of methylene blue solution varied in their ability to give a good blood stain, and that old solutions on which a scum had formed were best.

Present day blood stains are often spoken of as modified Romanovsky stains; altho the modifications are so great as to make them of a very different nature. The first modification was made by Nocht (1898) who concluded that the differential staining was due

*A good account of the history of these blood stains is given by MacNeal (1906).

to the formation of other dyes by the decomposition of methylene blue. Unna (1891) had already described what he called polychrome methylene blue, made by heating a solution of methylene blue on a water bath with potassium carbonate. Nocht decided to use this in the Romanovsky stain instead of untreated methylene blue. He found that it gave very good results if properly neutralized before mixing with eosin; and then learned that better results could be obtained by the use of a smaller amount of alkali and a longer period of polychroming, without subsequent neutralization.

The next step in preparing blood stains was made by Jenner (1899) who collected the precipitate formed when methylene blue and eosin are mixed, and redissolved it in methyl alcohol. He did not use polychrome methylene blue, and his stain lacked the nuclear staining principle of Romanovsky's and Nocht's stains; but it was an important step in that he showed the possibility of collecting the precipitated compound stain and of dissolving it in some solvent other than water. Combining this procedure with the Nocht stain was the next logical step and was taken independently by Reuter (1901) and by Leishman (1901). The method thus introduced was briefly to follow Nocht's technic of combining eosin with polychrome methylene blue, but then to filter off the precipitate and to redissolve it in methyl alcohol, not adding further water until the moment of applying the stain to the blood films.

Modern blood stains are in general modifications of Leishman's, differing only in detail. Wright's modifications, the one most used in America, (see Mallory and Wright, 1924, p. 170) differs from Leishman's only in that he prepared polychrome methylene blue by heating for only an hour in flowing steam, whereas the Leishman technic calls for twelve hours at 65°, with subsequent standing for ten days. Balch's modification calls for a polychrome methylene blue prepared by standing ten days with precipitated silver oxide.

Giemsa's and MacNeal's modifications are somewhat different. Giemsa obtained methylene azure, in what he considered a pure form, and combined it with eosin in order to obtain a more definite compound than when polychrome methylene blue is used. Then to obtain better differentiation he mixed it with methylene blue. Following his instructions, the Grübler Co. put on the market a product known as Azure II, which was a mixture of Azure I (i.e., methylene azure) and methylene blue in equal parts; and also a compound known as Azure II-eosin, which was an eosinate of Azure II, or more precisely a mixture of the eosinate of methylene blue with that of Azure I, in equal parts. This latter compound is the one generally known as the Giemsa stain. MacNeal (1922) proposed a method for obtaining a very similar blood stain, pre-

pared on even more scientific principles. This stain, known as the tetrachrome blood stain, is prepared by mixing definite proportions of methylene blue, methylene violet, methylene azure, and eosin. When first proposed (1922) this stain was to be prepared with a crude methylene azure, the pure product being at that time difficult to prepare and therefore expensive. MacNeal's latest work, however (1925), shows a simple method of preparing methylene azure A (asymmetric di-methyl-thionin), which is apparently the important ingredient of Azure I, and he now specifies azure A in the tetrachrome stain instead of the less definite product methylene azure, as formerly. Azure A for this purpose can already be obtained on the market in America.

Some difficulty was experienced at first in compounding the eosin-methylene-blue blood stains when imported dyes were no longer available. In some cases these difficulties were probably due to poor methylene blue or to poor eosin; but upon investigation the solvent, rather than the dyes themselves, has been found to be most often at fault. As stated above, the precipitated compound dye must be dissolved in methyl alcohol; but there are many grades of methyl alcohol and not all are equally suitable for the purpose. Apparently absolute purity is not needed; but two points are very important; the methyl alcohol must be neutral in reaction, and it must be free from acetone. In specifying a methyl alcohol for the blood stains, these two properties should be insisted upon. Very good methyl alcohol for this special purpose is now on the market.

Other Compound Stains

Various other compounds of acid and basic dyes have been used for special purposes. The basic dye employed in these compounds is ordinarily methyl green or methylene blue; but sometimes basic fuchsin, pyronin or rhodamine is used. The most commonly used acid dyes are eosin, orange G and acid fuchsin; but certain others are occasionally employed. Picric acid forms a few useful compound dyes, rosanilin picrate (i.e., the compound of basic fuchsin and picric acid) being especially well known as a tissue stain.

The Pappenheim panoptic triacid stain is a modification of Ehrlich's triacid compouud. In this combination methylene blue or methylene azure is substituted for methyl green. It is a tissue stain of use in certain special technics.

Ehrlich has proposed various other neutral stains, the best known being a compound of acid fuchsin and methylene blue used for staining blood; and a compound of narcein, an acid dye, with two basic dyes pyronin and methyl green or methylene blue.

CHAPTER IX

THE NATURAL DYES

AS STATED above (p. 11) the group of natural dyes is shrinking as more and more of them are being produced by artificial means. Alizarin, for example, used to be a natural dye of much importance; but now the artificial manufacture of this dye is much more economical. The group of natural dyes, as ordinarily recognized, contains only those which are not yet produced by artificial means. Indigo, however, is listed in this chapter, because in its chemistry it does not fall in well with any of the groups of artificial dyes. Indigo is still obtained from the indigo plant, altho under present-day conditions its artificial manufacture is ordinarily the more economical.

The chemistry of the natural dyes is less definitely known than that of the artificial dyes. This is easily understood; for it will be recalled that there are two ways of obtaining information as to the chemistry of unknown compounds: the first by decomposing them into simpler compounds of known composition; and the second by manufacturing them from known compounds. In the case of dyes not yet prepared artificially the second of these two lines of procedure is out of the question; hence the difficulty in learning their exact chemical structure.

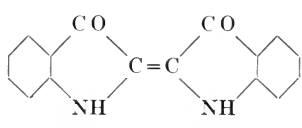
The most important natural dyes for the biologist are haematoxylin, indigo, cochineal (and its derivatives), orcein, and litmus.

The Indigo Group

INDIGO C. I. NO. 1177

Synonym: *Indigo blue.*

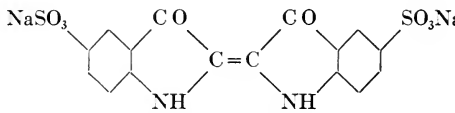
The plants from which indigo was formerly exclusively manufactured are largely species of the genus known as Indigofera, altho some indigo-bearing plants are recognized by botanists as belonging to different genera. In these plants is a glucoside, indican, which is converted by fermentation into the dye indigo. Various formulae have been given for indigo; the one favored at present is based upon its method of artificial manufacture:



In this formula the exact chromophore group is uncertain; but the ketone group (CO) in a closed ring occurs so often in dyes that it is regarded as probably having chromophoric properties.

Synonym: *Indigotine Ia.*

This is the sodium salt of indigodisulfonic acid:



Indigo carmin is a blue dye of acid properties, which is sometimes used as a plasma stain in contrast to carmin, either mixed with it or following it.*

Cochineal Products C. I. NO. 1239

Cochineal is a dye that has long been well known. It is obtained from a tropical insect generally known as the cochineal insect. By grinding and extracting the dried bodies of the female of the species in question a deep red dye is obtained, which is known as cochineal. By treating with alum this solution yields a product somewhat more free from extraneous matter, known as carmin. This is the form in which the dye is generally obtained by the microscopist. Cochineal products are used in various ways in microscopic technic, generally as nuclear dyes. They are extremely valuable in cases where it is desirable to stain in bulk before sectioning.

Cochineal, itself, has been used for various purposes in microscopic technic, even tho less used today than carmin. Alone it has little value, to be sure, for it has no direct affinity for tissues unless they contain iron, aluminium or some other metal. It is most commonly employed either with or following one of these mordants. A tincture of cochineal, that is an alcoholic solution containing calcium and aluminium chlorides, has been used by Mayer both on sections and for staining in bulk; but its most common method of use is with alum in watery solution. An alum-cochineal of this sort was first used independently by Mayer, Czokor, and Partsch; it can be used for sections, and is specially recommended for staining in bulk, by which technic it stains nuclei violet red, and blood and muscle cells orange, while the cytoplasm is but weakly colored. A chrom-alum-cochineal has been used by Hansen for staining sections. Spuler recommends an iron-alum-cochineal for staining in bulk when the sections are to be photographed, the technic bringing out nuclei, the blood in the tissues, and the muscle striations; sections may also be stained by the same method. By this technic the iron alum is applied first to the tissues as a mordant, and then followed by the stain. In Hansen's ferri-cochineal, on the other

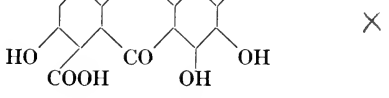
*For literature references to the procedures listed in this chapter see pp. 110 to 128 and 138 to 145.

hand, the iron alum is mixed with the dye, and the mixture used for staining sections of tissue.

Carmin is of considerable historic interest. It was used as early as 1839 by Ehrenberg, altho as we have seen (p. 7) not exactly for histological purposes. It was also employed in 1849 by Göppert and Cohn, by Corti in 1851, and by Hartig in 1854–8, these being the first uses of dyes in histology. It is still a valuable stain today, in spite of the enormous variety of synthetic dyes now available. On account of its freedom from toxicity it is useful for staining by injection. It is much used for staining in bulk, particularly in embryological work. A well known formula is Schneider's aceto-carmin, which is a valuable chromatin stain for fresh material in smear preparations. Alum carmin was used by Grenacher for similar purposes. Carmin is only slightly soluble in water at a neutral reaction; so solutions must be either acid (like the two above) or alkaline. Three alkaline formulae are of considerable use: ammonia carmin, which has been used both for injection and for staining sections; soda carmin, used primarily for injection; and Mayer's magnesia carmin, useful either for sections or for staining in bulk. Alcoholic solutions are also used: Grenacher's borax carmin (or as modified by Mayer) being a splendid nuclear stain for sections; and the hydrochloric carmin of Mayer serving both for sections and for staining in bulk. A special formula containing aluminium chloride (known as muci-carmin) has been proposed by Mayer and is used for staining mucin. In double staining it is sometimes used with indigo carmin; but most often with picric acid. Picro-carmin is a very well known combination used for double staining effects in sections, particularly for nervous tissue; it stains nuclei red and cytoplasm yellow.

One of the most recent and important uses of carmin is in Best's carmin stain for glycogen. The method is simple and the result beautiful, the red glycogen standing out in sharp contrast to the blue of the nuclei after staining in alum haematoxylin. The stain is permanent; the method is of much importance both to the pathologist and to the histologist.

Carminic acid. The dye principle of carmin and cochineal is carminic acid. This product is obtained by extracting the insect bodies with boiling water, treating the extract with lead acetate or barium hydrate, and then decomposing the lead or barium carminate with sulfuric acid. The exact composition of carminic acid is still somewhat uncertain; so far as known, it is:



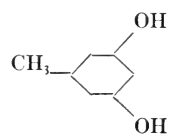
It is a fairly strong dibasic acid and forms readily soluble salts with the alkali metals, and insoluble salts with the heavy metals. Aluminium carminate (obtained by precipitation from aluminium acetate and carminic acid or ammonium carminate) is soluble in aqueous or weak alcoholic solutions of acids.

A slightly different aluminium compound, formed by mixing alum and carminic acid is used in histology. This combination was called carmalum by Mayer, and has also been used by Grenacher and Rawitz; it is a useful nuclear stain for sections, and is often employed with light green or indigo carmin as a contrast stain. A so-called muci-carmin, an acid solution containing aluminium chloride, has beem employed by Rawitz to stain mucin; while Mayer's para-carmine, containing aluminium and calcium chlorides, is used both for sections and for staining in bulk. By others a combination of iron with carminic acid has been used for similar purposes.

Carmein. Carmin, kept in ammoniacal solution, changes in its properties, due to oxidation. The oxidized carmin, often known as carmein, can be obtained by treating a carmin solution with hydrogen peroxide and precipitating with alcohol. It is a dark colored mass which can be ground into a black powder.

Orcein and Litmus C. I. No. 1242

Both orcein and litmus are obtained from certain lichens, *Lecanora tinctoria* and *Rocella tinctoria*. These lichens are colorless, but when treated with ammonia and exposed to the air, blue or violet colors develop. The colors are due to certain acids, one of which is orcin:



Orcin, acted upon by air and ammonia, becomes orcein.

ORCEIN

The exact formula of orcein is unknown. It is a weak acid, soluble in alkalies, with a violet color.

In alcoholic solution Unna has used orcein for staining elastin tissue; he has employed it for connective tissue, following polychrome methylene blue; and for plasma fibrils in the epithelium, following anilin blue; also with anilin blue or acid fuchsin in studying the process known by him as chromolysis. It has found less frequent use among other histologists; but has been employed by

Israel in acetic acid solution for staining sections (nuclei staining blue, cytoplasm red); and by Moll, dissolved in weak hydrochloric acid, for staining sections of embryos.

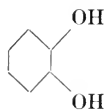
LITMUS

The exact composition of litmus is likewise unknown. It is obtained from the same lichens as orcein, treating them with lime and potash or soda, in addition to air and ammonia. Its colored principle is known as azolitmin.

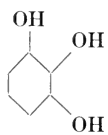
Litmus scarcely needs comment here. It is a feeble dye and is never used as an histological stain. Its classic use is for indicator purposes; but it is now coming to be largely replaced by the various synthetic dyes (especially sulphonphthaleins) which change color thru an hydrogen-ion range near the neutral point.

Brazilin and Haematoxylin

The two natural dyes, haematoxylin and brazilin are closely related chemically and upon decomposition yield the two compounds, pyrocatechin



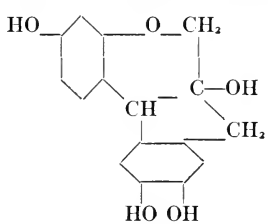
and pyrogallic acid



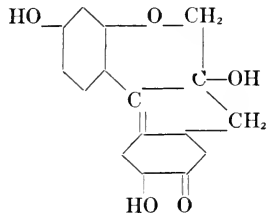
Both dyes are obtained by extraction of the bark of certain trees, haematoxylin from logwood and brazilin from brazil wood (red wood). Both trees are legumes and belong to the family Cesalpiniaceae; they are found only in the tropics. Haematoxylin comes from a single species; while brazil wood is a term applied to various different species all yielding brazilin.

BRAZILIN C. I. NO. 1243

The composition of this substance is supposed to be:



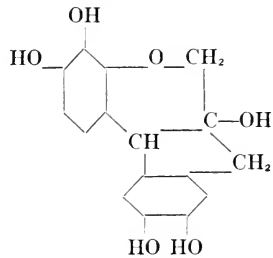
Its solution is colorless, but it becomes red on exposure to the air, as it is then oxidized into the dye brazilein, which probably has the formula:



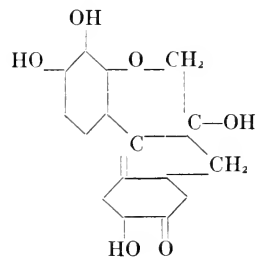
With alum it is used as a nuclear stain (known as brazalum) by Mayer. It is also used by Hickson for similar purposes following treatment with iron alum as a mordant.

HAEMATOXYLIN C. I. NO. 1246

Haematoxylin is a homolog of brazilin, having one more hydroxyl group, the generally accepted formula being:



Like brazilin it is not a dye, but its color develops in solution upon standing, due to the oxidation into haematein, which is homologous to brazilein and probably has the formula:



Haematoxylin is without question one of the most important biological stains. It is as valuable to the cytologist and histologist as methylene blue is to the bacteriologist; and probably is second

only to methylene blue in the number of different purposes for which it is used. It is valuable not only because it is a powerful nuclear stain and a chromatin stain *par excellence*, but also because it has striking polychrome properties. With the proper differentiation it is possible to get several shades intermediate between blue and red to show in the same preparation.

Haematoxylin is seldom used alone, as it has little affinity for the tissues in itself, even after "ripening" when it is largely converted into haematein. Some form of mordanting is ordinarily required; and most of the haematoxylin formulae either call for some metallic salt or specify previous treatment of the sections with one. In plant histology, however, there is some use for haematoxylin alone. Its greater affinity for plant than for animal tissue implies the presence of aluminium, copper, or iron in the former. In fact haematoxylin can be used as a very delicate reagent for iron or copper.

Perhaps the best known formulae for staining with haematoxylin are the combinations with aluminium, generally in the form of alum. Böhmer's alum haematoxylin (1865), altho no longer used, is of much historic interest as it was the first stain of this type to be used. The best known at present is Delafield's alum haematoxylin, which is very useful tissue stain with great affinity for chromatin and nuclei, and has much value in staining cellulose walls in vascular plants. Another alum haematoxylin used for similar purposes is that of Ehrlich.

Mayer's haemalum is another well known alum combination. In this stain haematein is first prepared and then combined with alum. The name haemalum, proposed by Mayer, is now generally accepted for this combination, and various other haemalum formulae have since been proposed. They are useful chromatin stains and are called for in various special procedures.

Mayer has also combined haematein with aluminium chloride, his haemacalcium calling for this salt and calcium chloride, while his muc-haematin contains aluminium chloride and glycerin. The latter is used for staining mucin.

The iron combinations are perhaps equally valuable. The original iron haematoxylin was that of Benda; but the best known at present is M. Heidenhain's, which is one of the most useful histological and cytological stains, both in botany and zoology. It is a powerful stain for chromosomes and centrosomes, and is of use for bringing out the middle lamellae in wood. Various other modifications of iron haematoxylin have been used, but they are all similar in principle. Ordinarily the iron salt is not mixed with the stain, but is used for a preliminary mordanting of the tissue.

Haematoxylin has been combined with chromium, one of the early staining methods being that of R. Heidenhain, which called for potassium bichromate as a mordant. Various recent modifications are in use today, such as that of Apathy, for staining general

tissue. Weigert uses a chrom combination for staining nervous tissue.

Benda uses haematoxylin following treatment with a copper salt for studying spermatogenesis; and Bensley a similar technic for chromosomes and mitochondria.

Mallory has proposed a formula for haematoxylin containing phosphomolybdic acid and also one containing phosphotungstic acid. The latter method is especially valuable for staining cells in the process of mitosis, and for distinguishing fibroglia, myoglia and neuroglia fibrils from collagen and elastin fibrils, especially in tumors, but also in normal tissues. It brings out sharply the striations in skeletal and cardiac muscle fibers. Haematoxylin is used in combination with other stains, especially eosin, but not so frequently as in the case of the common anilin dyes. The Van Gieson technic calls for haematoxylin followed by picric acid and acid fuchsin. A few other methods call for picric acid or ammonium picrate after haematoxylin; and it is sometimes used with eosin or after orange G or acid fuchsin. Most of these combinations, however, are called for only in the case of special procedures.

At present it is possible to obtain very satisfactory haematoxylin manufactured in America. A very good statement of the situation is given by McClung (1923). The first American haematoxylin put on the market, altho pronounced very good by many of those using it, still proved to be lacking in some of the qualities which the best haematoxylin ought to possess. Thru the coöperation of the manufacturers, the trouble was located. In order to obtain a pure product, SO_2 had been used in the manifacture for bleaching purposes; but this treatment was found to be harmful to the haematoxylin. The product now on the market is not so treated, however, but an extra recrystalization is introduced in the place of the bleaching process; and the resulting product meets all the tests to which it is submitted.

CHAPTER X

THE THEORY OF STAINING

THRUOUT the preceding pages of this book an effort has been made so far as possible to avoid theoretical discussions. Altho they contain some statements the truth of which cannot be regarded as fully established, as in the case of the chemical composition of some of the dyes, the discussion in general has been confined to observations and to chemical information for which there is good authority, without any attempt to introduce explanations of a theoretical nature. The present chapter, therefore, was not part of the original plan of the book, and the decision has finally been made to introduce it merely because it is felt that a brief statement of some of the most probable theories to explain staining may be of value in assisting the histologist in the intelligent use of stains for his purposes.

A long theoretical discussion of this subject might be included here, basing it upon the lengthy arguments supporting the various theories that have appeared in the literature. Such a detailed discussion, however, would probably be of little value. Hence this chapter is confined to a bare outline of the important points of the different theories.

Theoretically the dyeing of textile fabrics and the staining of microscopic structures are the same. In one case only the gross effects are observed, in the other the microscopic details. Any theory, therefore, that will explain the details of microscopic staining will be fully adequate to account for dyeing in bulk.

Theories to account for dyeing or staining have in general been based exclusively upon either physical or chemical phenomena. It would seem at first thought that the dyes combine so firmly with the tissues stained by them that the phenomenon must be a chemical one; but the exponents of physical theories have taken pains to show that all the observed facts can be explained on a physical basis, and that some observations are hard to explain if a chemical union between tissue and dye actually takes place. In a chemical union a new substance is formed which does not necessarily have the properties of either substance entering into its formation, and it is ordinarily impossible to recover the original substances by means of simple solvents. When tissue is stained there is no evidence of any new substance having been formed, the colored tissue merely taking on one of the characteristics of the dye (color) in addition to the properties which it originally possessed; it is, moreover, ordinarily possible to extract all or nearly all of the color by sufficiently long immersion in water, or by the fairly brief action of alcohol. Another observation which points against

chemical action is that the tissue never removes the dye completely from solution, even tho very dilute; whereas ordinary chemical reactions tend to continue until one of the components of the reaction is exhausted. Such facts as these, to the exponents of the physical theory, are enough to refute the possibility of chemical action.

It has, indeed, been pointed out that all of the ordinary dyeing or staining phenomena can be explained on a physical basis. It is evident, to be sure, that the action of the dyes is not confined to the surface of the material colored; but as the substances stained are always more or less porous, absorption of the dye after passing thru cell membranes by osmotic action accounts for the penetration. To account for the selective action of different dyes upon different parts of the cell it is possible to use the principle of absorption as an explanation. Adsorption is the property possessed by a solid body of attracting to itself by purely physical means from a surrounding solution certain compounds or ions present in that solution. Having once entered the tissue it is possible that the dyes remain there in a state of solid solution, similar to that in which gold is retained in ruby glass. This possibility, in the opinion of those who hold the physical theory, is the more likely because a dye causes the tissue to become the same color as the dye shows in solution, but not necessarily the same as it shows in its solid form. Dry fuchsin, for example, is green; its solution, however, is red, and so are tissues stained by it, no matter how completely they may be dried.

Some of those who hold in general to the physical theory of staining admit that these simple physical phenomena alone cannot explain everything, as for example, instances in which a dye penetrates different cell elements equally readily, but can be easily extracted from some of them while scarcely at all from others. It is assumed, therefore, that the dyes penetrate the cells by mere absorption and diffusion, but are in some cases precipitated there by acids or bases, or other chemical reagents present, thus preventing their extraction by simple solvents. Such a theory admits the possibility of chemical action without assuming an actual chemical union between the dye and the tissue.

In this connection the action of mordants is interesting. Some tissues do not stain directly with certain dyes, or if they do the color is very feeble. If, however, they are treated previously or simultaneously with certain chemicals, the dyes "take."

It is possible that this phenomenon may be due to chemical affinities of the mordant for the tissue on the one hand and the dye on the other; but the special value of iron and aluminium salts as mordants makes it seem quite probable that their action may be actually to precipitate the dye in the tissue. To the bacteriologist the behavior of different bacteria to the Gram stain immediately suggests itself. In this technic (see p. 68) one of the methyl violet dyes is allowed to act on the bacteria for a definite length of time,

and is then followed by treatment with iodine. After that, alcohol is applied for decolorization; but it proves that certain kinds of bacteria retain the violet stain even after counterstaining with some dye of a different color; while others are readily decolorized by the alcohol, and take the counterstain. According to some, this action is accounted for by assuming that the iodine combines with the methyl violet inside the cell, converting it into a molecule so large as to be unable to pass thru the cell membrane again. It may, on the other hand, be assumed that there is an actual chemical difference between Gram-positive and Gram-negative bacteria, so that the former combine chemically with the iodine and dye, while the latter do not. Neither theory has been definitely proved or disproved.

Evidence is still lacking, in fact, to prove or disprove either the chemical or the physical theory as it relates to general staining. The difference, perhaps, is not one of immense importance. It is frequently pointed out that there is no sharp distinction between chemistry and physics, and in such delicate reactions as those involved in staining, we may be well in the borderland between the two branches of science, where it is impossible to say that a given phenomenon is purely physical or purely chemical. There are, however, certain chemical principles distinctly different from the physical ones just mentioned, that may well enter into the phenomenon of staining; and it is these that are considered most important by the exponents of the chemical theory.

It is pointed out on behalf of the chemical theory that just because physical forces alone can explain the facts, one is not justified in assuming that chemical unions do not take place when the opportunity for them is present. It is known that some parts of the cell are acid in reaction, others alkaline; and it is a well known chemical principle that the former would tend to combine with the kations in solutions with which they come in contact, the latter with the anions. Now inasmuch as in certain dyes the color exists in the kation (basic dyes) and in others in the anion (acid dyes), it is natural to expect chemical combinations to take place between dye and tissue, depending upon the reaction of the latter. Arguments for the physical theory which exclude chemical action must furnish strong proof that no chemical union occurs; and those who favor the chemical theory claim that such proof is lacking. That the stained tissue does not present any characteristics to the eye not possessed by either tissue or dye before staining does not prove that no new substance has been formed, nor is this claim proved by the fact that sufficiently long action of solvents removes the color. Alcohol and even water are not absolutely inert chemically and may withdraw the dye by chemical instead of physical action; the very length of time necessary to remove the color completely (sometimes so long as to allow bacterial decomposition of the tissue) indicates that a rather strong union between dye and tissue has

taken place. As to the fact that a tissue which has a strong affinity for some particular dye never withdraws that dye completely from a very dilute solution, those who favor the chemical theory point out instances where chemical reactions are known to take place and yet to stop before either component is exhausted; and they further claim that chemical action is strongly indicated by the fact that in dilute solutions the tissues take up relatively larger quantities of the dye than in concentrated solutions.

In brief, the chemical theory of staining is that the tissues have certain definite chemical affinities which are satisfied by the chemical affinities of the dyes; tnerefore, when the tissue is put in a solution of the dye the latter combines with those portions of the tissue or of the individual cells which have the proper chemical nature. This theory, it will be seen, is especially well adapted to explain the differential staining which takes place, as when we find a certain stain acting only on tde nuclei or even exclusively upon certain structures within the nuclei. The chemical theory is not yet firmly established, and the probabilities are that staining is both a chemical and physical phenomenon; but it is coming to be more widely accepted than the physical theory. Hence it deserves a more detailed discussion.

The chemical theory of staining is dependent largely upon the question of the acid or the basic character of the dye molecule. It will be recalled that all ordinary dyes are encountered either as sodium or potassium salts of dye acids or as dye salts of colorless acids, the former being the acid dyes and the latter the basic dyes; while certain compound stains are neither acid nor basic dyes, inasmuch as the property of color exists in both the anion and the kation.

Briefly stated, the fundamental chemical theory of staining is that certain parts of animal or plant cells are acid in character and hence have an affinity for the basic dyes. The nuclei of the cells, or especially the chromatin within the nuclei, are assumed by this theory to be acid in character (due largely to their constituent nucleic acid), and there is no question but that they have a strong affinity for basic dyes; while the cytoplasm has an affinity for acid dyes and is assumed to be basic in character.

Now this is by no means the whole of the chemical theory of staining. The theory is so complex and has so many ramifications and special applications that it takes an intensive study of the subject to comprehend it fully. It is well known for instance, that certain basic dyes have stronger affinities for certain parts of the nuclei than for others, and that of the various cytoplasmic structures outside of the nucleus some are more readily stained by certain acid dyes and some by others. Such special applications as these, of course, are not explained on the theory of acid or basic character alone. It is possible, for example, to use the Flemming triple stain, which employs the acid dye orange G and the two basic

dyes safranin and gentian violet, thus staining the chromatin with gentian violet and the rest of the nucleus with safranin. It is difficult to say just how any chemical theory of staining can yet satisfactorily explain such selective action as this. It is, indeed, admitted by some upholders of the chemical theory that the chemical action of dyes is not specific, and merely serves to differentiate acid from basic elements of the tissue; and that the further differentiation, as between chromatin and other parts of the nucleus, is due to physical forces, thus indicating a difference in the structure rather than in the chemistry of the different portions of the nucleus.

The neutral stains have a very interesting action. When tissue is stained with them they seem to break up partly into their component acid and basic elements and stain portions of the tissue as the simple dyes would individually. But in addition the neutral stain itself seems to have an affinity for certain parts of the tissue and hence a third color is possible. This explains in part the polychrome effects obtained by the eosin-methylene-blue blood stains—only partly so, however, because in preparing these stains methylene blue breaks up into certain other dyes so that more shades than those expected from eosin and methylene blue alone are obtained.

So far as the action of stains is chemical, their use forms a connecting link between the two subjects, histology and microchemistry. These two branches of science are generally thought to be entirely distinct. The histologist, with the technic and viewpoint of the biologist, prepares delicate sections of various materials colored with one or more of a long series of available dyes, and studies the biological structures present under the microscope. The microchemist, with the technic and viewpoint of the chemist, examines with the microscope similar material treated with various reagents of known chemical reaction, and from his observations draws conclusions as to the chemical nature of the substances examined. There is some possibility, however, that the difference between histology and microchemistry is one of point of view rather than of methods. Both the microchemist and the histologist study the action of chemical compounds on substances or structures visible under the microscope; the difference is that the microchemist uses the chemical compounds in question as chemical reagents while the histologist uses his as dyes to color the microscopic structures and thus to increase their visibility.

Now, on the chemical theory of staining, the biologist is using complicated chemical reactions in his microscopic technic—so complicated in fact as to be unintelligible chemically. To bridge the gap between histology and microchemistry these reactions must be made intelligible. The first step in this direction has now been taken by Unna (1921) in a very important contribution to the subject of cell chemistry. He points out the need of harmonizing chemical and histological investigations and proposes a method of doing this which he calls *chromolysis*. The technic he has de-

veloped, altho quite complicated in its details, can be summed up briefly as follows: To select a dye, or a mixture of dyes, either acid or basic, which bring out some intracellular structure whose chemistry it is desired to learn; then to submit the sections to the action of various solvents, beginning with simple cold water, next proceeding to hot water, and from that to the more powerful solvents, but using only those whose action on proteins, lipoids or carbohydrates is known to the chemist; then to stain the sections with the staining fluid selected; and finally to determine by microscopic examination which solvents have removed the substance under investigation.

By such methods as these Unna hopes to make considerable progress in the microchemistry of the cell; and it will be readily seen that once the gap between chemistry and histology is bridged, progress will become constantly more and more rapid. As soon as it is possible to obtain reasonable hypotheses as to the chemical nature of the various intracellular bodies in any one particular type of tissue, then it will be possible to ascertain the affinities of the different dyes now used in histology for the different chemical compounds thus recognized; and then by using the same stains on other tissue it will be possible to apply the information thus obtained to the solution of the chemistry of other microscopic structures. In other words, stains will become chemical reagents instead of merely dyes for making microscopic structures visible. In this way it is hoped that chemistry and histology, working together, may solve some of the obscure problems as to the nature of the cell and its contents.

APPENDIX I

TABLES RELATING TO STAINS

In the following tables all the dyes that are frequently mentioned in the literature dealing with microscopic technic are listed, together with the most important uses of each in the biological laboratory. The list of uses (Table 2) is necessarily incomplete. In the case of the most commonly used stains, in particular, it has been necessary to group various uses together under some general term, without attempt to list the individual procedures. In general, however, the policy has been to list the methods for which a stain is most commonly used today, and of the obsolete methods to give only those of historical interest. Criticism will be welcomed from anyone noticing any serious omission.

The dyes in Tables 1 and 2 are listed in the same order as in the main part of this book. Hence either the general Table of Contents or the list in Table 1 may be used to learn the order of the stains when it is desired to find some particular one in Table 2. For an alphabetical list referring to the following pages see the general Index.

The references given in the last column of Table 2 refer ordinarily by name and date to the literature listed in Appendix III. Five references, however, are used so often that the date is omitted: they are referred to merely as *Chamberlain, Lee, Ehrl. I, Ehrl. II,* and *Mal. & Wr.* These refer respectively to: Chamberlain's Methods in Plant Histology (1924), Lee's Microtomists Vade-mecum (1921), Ehrlich's Enzykopädie der Mikroskopischen Technic (1910), Vol. I and Vol. II, and Mallory and Wright's Pathological Technic (1924). No effort has been made to give the original reference in all cases; but rather to refer to some readily available description of each technic than can be followed by anyone using the procedure.

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TABLE I. NOMENCLATURE AND SYNONOMY OF STAINS.

Colour Index No.	Schultz* No.	Preferred Designation	Synonyms; and slightly varying shades	Page reference
		(a) Synthetic Dyes		
7	5	Picric acid		32
9	6	Martius yellow	Napthol yellow. Manchester yellow	32
12	IV 2	Aurantia	Imperial yellow	33
27	38	**Orange G**†	Patent orange. Acid orange G, GG, GGO. Crystal orange GG. Wool orange 2G, (Slightly differing grade: Orange GG, GMP)	34
88	112	Bordeaux red	Fast red B, P. Cerasin. Archelline 2B. Azo-bordeaux, Acid bordeaux. (Various grades denoted as: Bordeaux B, BL, G, R extra).	35
133	124	Janus green B	Diazin green	35
16	137	Fast yellow	Acid yellow	35
142	138	Methyl orange	Orange III. Helianthin. Gold orange. Tropaeolin D	36
143	139	Orange IV	Orange N. Acid gold D. Tropaeolin OO	36
150	144	Orange I	Naphthol orange. Tropaeolin G, OOO No. 1	36
151	145	Orange II	Gold orange. Orange A, P, R. Acid orange. Orange extra. Mandarin G. Tropaeolin OOO No. 2	37
152	IV 104	Narcein		37
184	168	Amaranth	Naphthol red. Fast red. Bordeaux. Bordeaux SF. Victoria rubin. Azo rubin. Wool red.	37
248	223	**Sudan III**	Sudan red. Tony red. Scarlet G or B. Fettponceau G. Oil red. Cerasin red.	38
258	232	**Sudan IV**	Scarlet red. Fettponceau. Ponceau 3B. Oil red IV	38
280	247	Biebrich scarlet, water soluble	Croceine scarlet. Ponceau B. Double scarlet. Scarlet B, EC	39
331	283	Bismarck brown Y	Vesuvin. Phenylene brown. Manchester brown. Excelsior brown. Leather brown. (Slightly different shade: Bismarck brown G).	39
370	307	Congo red	Congo. Cotton red. Direct red. Cosmos red.	40
438	359	Trypan red		40
448	363	Benzopurpin 4B	Cotton red 4B. Dianil red 4C. Diamin red 4B. Sultan 4B. Direct red 4B.	40

477	391	Trypan blue	Diamin blue 3B. Benzo blue 3B. Dianil blue H3G. Congo blue 3B. Naphthamine blue 3BX. Benzamin blue 3B. Azidin blue 3B. Niagara blue 3B.	41
520	426	Niagara blue 4B	Niagara sky blue. Benzoin sky blue. Dianil blue H6G. Congo sky blue. Naphthamine blue.	41
1027	778	Alizarin	(Various grades denoted as: Alizarin P, VI, Ic).	42
1034	780	Alizarin red S	Alizarin monosulphonate of sodium. Alizarin red WS. Alizarin carmine.	43
1037	783	Purpurin	Alizarin No. 6. Alazirinpurpurin.	43
920	II 348	Thionin	Lauth's violet (Not thionin blue, which is Schultz No. 661).	45
922	659	**Methylene blue, Med. U. S. P.**	Swiss blue. (Slightly different grades: Methylen blue BX, B, BG and BB).	46
...	...	Methylene azure	Azure I	48
...	...	Methylene violet, Bernthsen	Not methylene violet RRA or 3RA, Schultz No. 680).	49
924	660	Methylene green		50
925	IV 592	Toluidin blue O	Methylene blue O.	50
927	663	New methylene blue N	Methylene blue NN.	51
877	621	Brilliant cresyl blue	Cresyl blue. Cresyl blue 2RN or 2BS. Brilliant blue C.	51
913	653	Nile blue sulfate	Nile blue A	52
...	...	Cresyl violet	Cresyl echt violet.	52
825	670	Neutral red	Toluylene red.	54
826	669	Neutral violet		54
841	679	**Safranin O**	(Slightly different shades: Safranin AG, T, MP, Y and G.)	55
847	686	Amethyst violet	Heliotrope B. Iris violet.	56
857	694	Magdala red	Naphthalene red, Naphthalene pink. Naphthylamine red. Sudan red.	56
865	700	Nigrosin, water soluble	Nigrosin W, WL, etc. Gray R, B, BB. Silver gray. Steel gray. Indulin black.	57
655	493	Auramin	Pyoktaninum aureum. Pyoktanin yellow. Canary yellow.	60
657	495	Malachite green	Emerald green. New Victoria green. Diamond green. Solid green. Light green N.	61

*The Schultz No. refers ordinarily to the sixth edition of Schultz's Farbstofftabellen (1923). When the number is preceded by a Roman numeral, the dye in question is not listed in the fifth edition and the Roman numeral indicates the edition in which it is to be found.

†The dyes printed in bold faced type are the most commonly used stains.

Colour Index No.	Schultz No.	Preferred designation	Synonyms: and slightly varying shades	Page reference
662	499	Brilliant green	Ethyl green. Malachite green G.	61
670	505	Light green SF, yellowish	Light green 2G, 3G, 4G, 2GN, Acid green (with various shade designations). Fast green N.	62
*676–8	511–3	**Fuchsin, basic**	Fuchsin. Magenta. Rubin. (Different grades sold under the name of fuchsin followed by various shade designations.)	63
692	524	**Fuchsin, acid**	Fuchsin S, SN, SS, ST or S III. Acid magenta. Acid rubin.	64
679	514	Hoffman violet	Dahlia. Iodine violet. Primula R. Red violet. Violet R, RR, 4RN.	65
680	515	**Methyl violet**	Dahlia B. Paris violet. Gentian violet. Pyoktanin blue. (Various shades denoted: Methyl violet 3R, 2R, R, B, 2B, 3B, BBN, BO, and V3).	66
681	516	**Crystal violet**	Violet C, G or 7B. Gentian violet. Hexamethyl violet. Methyl violet 10B.	67
...	...	**Gentian violet†**		68
684	519	**Methyl green**	Double green. Light green	69
686	II 284	Iodine green	Hoffman's green	71
689	521	Spirit blue	Anilin blue, alcohol soluble. Gentian blue. Night blue. Lyons blue. Paris blue.	71
706	538	Methyl blue	Cotton blue. Helvetia blue.	72
707	539	**Anilin blue, water soluble**	China blue. Soluble blue 3M or 2R. Marine blue. Cotton blue. Water blue. Berlin blue.	72
724	555	Rosolic acid	Aurin	74
724	555	Corallin, yellow	Sodium salt of rosolic acid.	74
739	568	Pyronin G		76
741	...	Pyronin B		76
749	573	Rhodamin B	Rhodamin O. Brilliant pink.	77
766	585	Fluorescein	Uranin	78
768	587	**Eosin, yellowish**	Eosin. Water soluble eosin. Eosin Y, W or WS. (Various grades denoted as Eosin G, Y extra, S extra, J extra, B extra, GGF, 3J, 4J, KS, DH, and JJF.)	79

769	588	Methyl eosin	Eosin, alcohol soluble.	79
770	589	Ethyl eosin	Eosin, alcohol soluble. Eosin S.	80
771	590	Eosin, bluish	Eosin BN, B, BW, DHV. Safrosin. Eosin scarlet B, BB. Scarlet J, JJ, V. Nopalin G. Caesar red.	80
772	591	Erythrosin, yellowish	Erythrosin R or G. Pyrosine J. Iodo-eosin G.	81
773	592	Erythrosin, bluish	Erythrosin B. Pyrosin B. Eosin J. Iodo-eosin. Dianthine B. (Slightly different shades: Erythrosin D, J, JNV, W).	81
774 or 778‡	593 or 596	Phloxine	Erythrosin BB. New pink.	82
777 or 779‡	595 or 597	Rose bengal	(Various grades denoted as: Rose bengal B, 2B, 3B).	83
			(b) Natural Dyes	
1177	874	Indigo	Indigo blue	91
1180	877	Indigo carmine	Indigotine Ia.	92
1239	932	**Cochineal**	(The aqueous extract of the cochineal insect).	92
		Carmin	(The lake prepared by adding alum to cochineal).	
		Carminic acid	(The active dye purified from cochineal).	
1242	934	Orcein		94
1243	935	Brazilin		95
1246	938	**Haematoxylin**	(The ether extract of logwood).	96
		Haematein	(The dye formed on oxidation of haematoxylin).	

*Three different dyes, corresponding to these three numbers both in Schultz and in the Colour Index, are apparently sold to biologists as basic fuchsin. It is not yet known which is the best or whether the different products may not be suited to different biological purposes.

†Nearly synonymous with methyl violet; various mixtures of methyl and crystal violets are sold under this name, generally with a high percentage of dextrin.

‡There are in each case two different dyes, as indicated by the Colour Index and Schultz numbers, denoted phloxine and rose bengal. It is still uncertain which one is generally furnished or which one is desired by biologists.

TABLE 2. THE PRINCIPLE USES OF THE MOST IMPORTANT STAINS

Name of stain and page reference*	Name or Author of Technic	Application	Literature references†
Picric acid p. 32		For cytoplasm. Contrast stain.	Lee, p. 176. Ehrl. II, p. 399
	Van Gieson	With acid fuchsin; for connective tissue.	Lee p. 176. Ehrl. I, p. 249. Ehrl. II, p. 474.
Martius yellow p. 32	Pianese	For cancer tissue; with acid fuchsin and malachite green. Same technic adapted for staining pathologic plant tissue (fungus diseases).	Pianese, 1896. Müller, 1912. Vaughan, 1914.
Aurantia p. 33		For light filters in photomicrography.	Ehrl. II, p. 68.
	Champy-Kull	For mitochondria; with acid fuchsin and toluidin blue.	Lee, p. 321.
Orange G p. 34		A valuable plasma stain in sections of tissue.	Ehrl. II, p. 327.
		Background stain for haematoxylin and other nuclear stains in botanical histology.	Chamberlain, p. 62
	Flemming's triple stain	As cytoplasm stain, in contrast to crystal violet and safranin.	Fleming, 1881. Lee, p. 177. Ehrl. I, p. 475–7. Ehrl. II, p. 327.
	Mallory's connective tissue stain	For cytoplasm and red blood cells; with anilin blue and acid fuchsin	Mallory, 1900. Mal. & Wr., p. 118. Ehrl. I, p. 49.

	Ehrlich-Biondi-Heidenhain.	For tissues; with methyl green and acid fuchsin.	Ehrl. I, p. 277–80. Lee, p. 172–3.
	Ehrlich triacid mixture.	For blood; combined with methyl green and acid fuchsin.	Ehrl. II, p. 313.
	Bensley's neutral gentian.	Combined with gentian violet; for islands of Langehans.	Bensley, 1911.
Bordeaux red p. 35		For cytoplasm, before staining with Heidenhain's haematoxylin.	Ehrl. I, p. 157.
	Gräberg.	For sections of spleen, testis and liver; with methyl green and thionin.	Ehrl. I, p. 157.
Janus green B p. 35		For vital staining of chondriosomes.	Michaelis, 1900. Bensley, 1911. Lee, p. 232–3.
		For sections of embryos; with neutral red.	Faris, 1924.
Fast yellow p. 35	Schaffer.	For bone sections	Schaffer, 1888. Ehrl. I, p. 267.
	Unna.	Combined with eosin, phloxine and anilin blue; in the study of chromolysis.	Unna, 1921.
Methyl orange p. 36	Bergonzini.	In place of orange G in the Ehrlich-Biondi stain.	Bergonzini, 1891. Ehrl. I, p. 550.
	Ebbinghaus.	For keratin in sections of skin.	Ebbinghaus, 1902. Ehrl. I, p. 550.
Narcein p. 37	Ehrlich.	As component of "neutral" stain mixtures.	Ehrlich and Lazarus, 1898, p. 25–7.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 138.

Name of stain and page reference*	Name or Author of Technic	Application	Literature references†
Amaranth p. 37	Griesbach.	For axis cylinders.	Griesbach, 1886.
Sudan III p. 38	Daddi.	For fat in tissues.	Daddi, 1896. Lee, p. 358–9. Ehrl. I, p. 445–53.
Sudan IV p. 38	Michaelis.	For fat in tissues.	Michaelis, 1901. Lee, p. 358–9. Ehrl. I, p. 445–53.
Biebrich Scarlet, W. S. p. 39		A general plasma stain.	Ehrl. I, p. 106.
	Pelagetti.	For cytoplasm, after polychrome methylene blue or Unna's haematein.	Pelagetti, 1904.
	Paladino.	Mixed with alum haematoxylin; for staining tissue sections.	Paladino, 1895.
Bismarck Brown Y p. 39		A general plasma stain, formerly much used. A good mucin stain. Good for vital staining and for staining in bulk.	Ehrl. I, p. 106. Ehrl. II, p. 489, 593.
		For bacteria, particularly in contrast to gentian violet in the Gram technic.	Hucker and Conn, 1923. Mal. & Wr., p. 273.
	Foot and Strobell.	Chromosomes in smear preparations of eggs.	Foot and Strobell, 1905.
Congo red p. 40		Background stain for haematoxylin and other nuclear stains.	Carnoy and Lebrun, 1897. Lee, p. 178, 408, 453. Ehrl. I, p. 255.

	Griesbach.	For axis cylinders.	Griesbach, 1886.
	Schaffer.	For sections of embryos.	Schaffer, 1888.
		For staining plant mucin.	
	Klebs.	Reagent for cellulose.	Klebs, 1886.
Trypan red p. 40		For vital staining.	Lee, p. 388–90.
Benzopurpin 4B p. 40	Zschokke	Plasma stain, especially in contrast to haematoxylin.	Lee, p. 179. Ehrl. I, p. 104. Zschokke, 1888.
		For vital staining.	Lee, p. 390.
Trypan blue p. 41		For vital staining.	Lee, p. 388–90.
Alizarin red S p. 43	Benda	For chromatin; in combination with crystal violet (chromatin brown, mitochondria violet).	Lee, p. 322. Ehrl. II, p. 196–9.
	Schrötter	For sections of nervous tissue.	Lee, p. 408.
		Vital stain for nerve tissues.	
Purpurin p. 43		A nuclear stain.	Ehrl. II, p. 446.
		Reagent for detecting insoluble calcium salts in protoplasm.	Ehrl. II, p. 446.
Thionin p. 45		For chromatin and mucin in general histology.	Lee, p. 168. Ehrl. II, p. 539.
		For frozen sections of fresh tissues.	

Name of stain and page reference*	Name or author of technic	Application	Literature references†
Thionin p. 45 (*continued*)	Ehrlich.	For amyloid (colored blue), mast cells and mucin (red).	Ehrl. II, p. 78.
	Gråberg.	With methyl green and bordeaux red, for sections of spleen, testis and liver.	Ehrl. I, 157.
	Frost	Staining very young bacterial colonies on "little plates" (i. e., thin layers of bacteriological media on microscopic slides.)	Frost, 1916.
Methylene blue p. 46		A widely used nuclear stain in general histology (zoological), with many special applications.	Lee, p. 186–96. Ehrl. II, p. 85–6.
		A favorite bacterial stain, used for many special purposes, as in examination of milk and diagnosis of diphtheria.	Ehrl. II, p. 87–8.
	Mallory	As nuclear stain, in contrast to eosin, on pathological or other histological material.	Mallory, 1904. Mal. & Wr. p. 100–2. Lee, p. 181.
	Ehrlich.	Staining small animals intra vitam. Vital staining of nervous tissue	Lee, p. 188–195. Ehrl. II, p. 593.
	Romanovski, and others.	For staining blood; in combination with eosin.	Ehrl. I, p. 121.
	Levine.	With eosin, as indicator in bacteriological media, for differentiating colon and aerogenes organisms.	Levine, 1921.
Methylene azure p. 48	Giemsa.	For staining blood, protozoa, etc.; in combination with eosin.	Ehrl. I, p. 122.
Methylene green p. 50		For wood and fixed chromatin, in plant sections.	

Toluidine blue O	Pappenheim's panchrome stain	A metachromatic nuclear stain often useful in place of thionin or methylene blue.	Ehrl. II, p. 542.
p. 50	Albert	Diagnosis of diphtheria	Albert, 1920.
Brilliant cresyl blue, p. 51		For blood; to bring out platelets and reticulated cells.	Robertson, 1917.
Nile blue sulphate, p. 52	Lorrain Smith.	For fats; to distinguish between acid and neutral fat.	Lee, p. 362.
Cresyl violet p. 52		For general histological tissue.	Lee, p. 184.
		For nervous tissue.	
		For fresh tumor tissue.	Williams, 1923.
Neutral red p. 54		For histological tissue (of embryos); contrast stain to janus green.	Faris, 1924.
		Nuclei, especially of blood cells, and Nissl granules of nerve cells, stained intra vitam.	Lee, p. 179. Ehrl. II, p. 317–8.
		"Vital staining" of blood, (i. e., fresh in moist chamber; also of fresh gonorrhoeal pus.	Lee, p. 382. Ehrl. II, p. 318–9.
		As indicator in bacteriological culture media; for distinguishing colon from typhoid organisms, and other similar purposes.	Rothberger, 1898.
Neutral violet p. 54	Unna.	Used in study of chromolysis.	Unna, 1921.
Safranin O p. 55		A widely used nuclear stain. (*See over.*)	Lee, p. 165–7, 352, 391. Ehrl. II, p. 476–8.

Name of stain and page reference*	Name or author of technic	Application	Literature references†
Safranin O p. 55 (*continued*)		For tissues of vascular plants, in combination with variety of contrast stains. Cutinized, suberized, and lignified tissue. Spore coats. Protein.	Chamberlain, p. 56, 88–90.
	Flemming's triple stain.	For chromatin and other nuclear elements, in conjunction with gentian violet; orange G as contrast stain.	Flemming, 1881. Lee, p. 177. Ehrl. I, p. 475–7. Ehrl. II, p. 327.
	Benda.	For chromatin; with light green.	Benda, 1891. Lee, p. 181.
	Flemming.	For bacteria, particularly in contrast to gentian violet in the Gram technic. For nuclei.	Hucker and Conn, 1923. Mal. & Wr., p. 273. Flemming, 1881.
Magdala red p. 56	Kultschitzky.	For elastic tissue.	Kultschitzky, 1895.
		For algae; with anilin blue.	Chamberlain, p. 58, 105.
Nigrosin p. 57		For central nervous tissue; alone or with other stains.	Ehrl. II, p. 322.
	Jarotzky.	For tissue of pancreas; following haematoxylin.	Ehrl, II, p. 322.
		For algae and fungi.	Chamberlain, p. 63.
	Unna.	Combined with "orange" (orange G?); used in study of chromolysis.	Unna, 1921.
	Pfitzer's picro-nigrosin.	For chromatin.	Pfitzer, 1883. Lee, p. 182, 350, 408.

Auramin p. 60	Fischel.	Vital staining of salamander larvae.	Fischel, 1901. Ehrl. I, p. 98.
	Vinassa.	For plant sections.	Vinassa, 1891. Ehrl. I, p. 98.
Malachite green p. 61	Pianese.	In combination with acid fuchsin and martius yellow; for cancer tissue.	Pianese, 1896. Mal. & Wr. p. 83.
		For host tissue in plants infected with fungi; used in the Pianese combination.	Müller, 1912. Vaughan, 1914.
	v. Beneden.	For Ascaris eggs.	Ehrl. II, p. 68.
	Petroff.	For erythrocytes.	Ehrl. II, p. 68.
	Maas.	Contrast stain, following borax carmine.	Ehrl. II, p. 68.
Brilliant green p. 61	Krumwiede.	Indicator in bacteriological media for differentiating organisms of colon, typhoid and dysentery groups.	Mal. & Wr., p. 228–9.
		For inhibiting *B. coli* in stools, and enriching *B. typhosus* in broth cultures.	Browning, Gilmore & Mackle, 1913. Torrey, 1913.
Light green SF, yellowish p. 62		A general plasma stain.	Ehrl. II, p. 33.
	Benda.	For spermatozoa; with safranin.	Benda, 1891.
		For cellulose walls in vascular plant tissue; contrast to safranin.	Chamberlain, p. 61.
		For general histological tissues; contrast to haematoxylin.	Peter, 1899. Prenant, 1902. Brazil, 1905.

Name of stain and page reference*	Name or author of technic	Application	Literature references†
Basic fuchsin p. 63		A powerful nuclear stain; with various green and blue contrast stains	Ehrl. I, p. 493–4.
		For mucin, fuchsinophile granules; for nuclear elements of central nervous system.	Id.
	Weigert.	For elastic tissue.	Weigert, 1898. Ehrl. I, p. 295.
		General bacterial staining.	Mal. & Wr., p. 272.
	Ziehl-Neelson.	For staining tubercle organism; diagnosis by acid fast property.	Ehrl. II, p. 349–50. Mal. & Wr., p. 393–8.
	Endo.	In bacteriological culture media for differentiating colon and typhoid organisms.	Endo, 1904. Mal. & Wr., p. 227–8.
Acid fuchsin p. 64		A widely used plasma stain.	Lee, p. 171, 321.
	Van Gieson.	In combination with picric acid; for connective tissue.	Lee, p. 176. Ehrl. I, p. 249. Ehrl. II, p. 474. Mal. & Wr., p. 80, 119.
	Mallory.	In combination with anilin blue and orange G; for connective tissue.	Mallory, 1900. Mal. & Wr., p. 118. Ehrl. I, p. 49.
	Ehrlich-Biondi-Heidenhain.	In combination with methyl green and orange G; for differential staining of sections and blood smears.	Ehrl. I, p. 277. Lee, p. 173–5.
	Ehrlich's tri-acid mixture.	In combination with methyl green and orange G; for blood smears.	Ehrl. II, p. 313. Lee, p. 175.
		For cortex, pith, cellulose walls, etc., in vascular plants.	Chamberlain, p. 57, 67
	Pianese.	For cancer tissue; with malachite green and martius yellow.	Pianese, 1896.

		Same technic adapted for staining fungus mycelium in infected plants.	Müller, 1912. Vaughan, 1914.
	Bensley-Cowdry.	For mitochondria; with methyl green.	Lee, p. 324.
	Andrade.	As indicator; especially in bacteriological media.	Mal. & Wr., p. 238.
Hoffman's violet p. 65	Ehrlich.	For mast cells.	Ehrl. I, 710.
	Juergens.	For amyloid in sections of tissue.	Id.
Gentian violet p. 68		A powerful nuclear stain.	Ehrl. I, p. 517.
		A very valuable stain in plant histology, particularly for the achromatic nuclear structures.	Chamberlain, p. 59–60
	Flemming's triple stain.	For nuclear structures in conjunction with safranin; orange G as contrast stain.	Flemming, 1881. Lee, p. 177. Ehrl. I, p. 475–7. Ehrl. II, p. 327.
	Weigert.	For fibrin and neuroglia in fresh tissue.	Weigert, 1881. Mal. & Wr., p. 150–1.
		For amyloid; in frozen sections of fresh tissue.	Mal. & Wr., p. 202.
	Bensley.	Combined with orange G, to form "neutral gentian:" for demonstrating islands of Langerhans.	Bensley, 1911.
	Gram.	As bacterial stain; generally with alcoholic differentiation to make it selective, and some red or brown dye as counterstain.	Gram, 1884. Hucker & Conn, 1923. Benda, 1899.
Crystal violet p. 67	Benda.	For mitochondria; with alizarin red.	Ehrl. II, p. 198. Lee, p. 322.
		For lightly lignified walls in plants; with erythrosin. (Proves more uniform than gentian violet).	

Name of stain and page reference*	Name or author of technic	Application	Literature references†
Methyl green p. 69		An excellent nuclear stain.	Lee, p. 159–61. Ehrl. II, p. 113.
		In weak acetic acid solution, for fresh chromatin.	Lee, p. 293–4.
		For lignified xylem in plants; with acid fuchsin.	Chamberlain, p. 61.
	Galeotti.	As cytoplasm stain; following acid fuchsin and picric acid.	Ehrl. II, p. 113.
	Ehrlich-Biondi-Heidenhain.	In combination with acid fuchsin and orange G; stains nuclei in sections and in blood.	Ehrl. I, p. 277. Lee, p. 173–5.
	Bensley-Cowdry.	For chromatin; in contrast to acid fuchsin, which stains the mitochondria.	Lee, p. 324.
	Ehrlich's tri-acid mixture.	In combination with acid fuchsin and orange G; for blood smears.	Ehrl. II, p. 313. Lee, p. 175.
	Pappenheim.	In combination with pyronin; for gonococcus, and for mast cells.	Pappenheim, 1899. Ehrl. II, 447. Lee, p. 172.
Iodine green p. 71	Griesbach.	As nuclear stain.	Griesbach, 1882.
		For mucin and amyloid.	Stilling, 1886. Ehrl. I, p. 46.
		For lignified xylem in plant sections; with acid fuchsin.	Chamberlain, p. 61, 67
	Zimmermann.	For chromatin in plant tissue; with acid fuchsin for nucleolus and spindle fibres.	Zimmermann, 1893 Ehrl. I, p. 709.
	Ciaccio.	For nervous tissue; with acid fuchsin and picric acid.	Ciaccio, 1906.
	Lefas.	For blood; with acid fuchsin.	Ehrl. I, p. 709.

Methyl blue p. 72	Mann.	For nerve cells; with eosin.	Lee, p. 183. Ehrl. I, p. 357.
	Dubreuil.	Combined with picric acid, as tissue stain; followed by carmalum or safranin.	Ehrl. II, p. 84.
Anilin blue, water soluble p. 72		A good plasma stain, used particularly in nervous tissue and cartilage.	Lee, p. 183. Ehrl. I, p. 49.
	Mallory.	For connective tissue; with orange G and acid fuchsin.	Mallory, 1900. Mal. & Wr., p. 118. Ehrl. I, p. 49.
	Stroebe-Huber.	As cytoplasm stain, preceding safranin.	Ehrl. I, p. 49.
	Unna.	For sections of epithelium; with orcein.	Lee, p. 340.
Pyronin p. 76	Pappenheim.	In combination with methyl green; for bacteria (especially in organic liquids), mast cells and other basophile elements.	Pappenheim, 1899. Lee, p. 172. Ehrl. II, p. 447.
	Ehrlich.	In combination with narcein and methyl green or methylene blue, for blood, etc.	Ehrlich and Lazarus, 1898.
		For bacteria; sometimes used as counterstain to gentian violet in the Gram technic.	Mal. & Wr., p. 273.
Rhodamine B or S p. 77	Griesbach.	With osmic acid; to fix and stain blood simultaneously.	Ehrl. II, p. 470.
		As plasma stain, in contrast to methyl green or methylene blue.	Id.
	Ehrlich.	As a component of certain "neutral" stain mixtures; for blood, etc.	Id.

Name of stain, mordant, etc. Also page ref.	Name or author of technic	Application	Bibliographic references
		Very useful cytoplasm stain.	Ehrl. I, p. 355–8.
	Mallory.	As cytoplasm stain in contrast to methylene blue, on pathological or other histological material.	Mallory, 1904. Mal. & Wr., p. 100–2.
		For algae and fungi; counterstain to haematoxylin, etc.	Chamberlain, p. 57–8.
Eosin Y, p. 79	Mann.	In combination with methylene blue; for sections of tissues.	Ehrl. I, p. 357.
	List.	Cytoplasm stain; preceding methyl green.	List, 1885.
	Teichmüller.	For sputum; preceding methylene blue.	Teichmüller, 1899.
	Romanovsky, etc.	In combination with methylene blue; for blood smears.	Ehrl. I, p. 121. Mal. & Wr., p. 470–3.
Eosin, alc. sol., p. 97		Counterstain following Delafield's haematoxylin in general animal histology.	
	Held.	Cytoplasm stain for nerve cells; preceding methylene blue.	Ehrl. I, p. 358.
Erythrosin, p. 81		Counterstain for sections of vascular plants; in contrast to haematoxylin, gentian violet, etc.	Chamberlain, p. 58.
	Winogradski.	Stain for bacteria in dried smears of soil suspensions.	Winogradski, 1924.
Phloxine, p. 82	Chamberlain.	For staining algae.	Chamberlain, p. 59.
	Unna.	Combined with various other acid dyes; for use in studying chromolysis.	Unna, 1921.

Rose bengal, p. 83		Cytoplasm stain, following haematoxylin.	Pelagetti, 1904.
	Conn.	Stain for bacteria, especially those forming slime; also for dried smears of soil suspensions.	Conn, 1921.
Indigo carmin, p. 92		As plasma stain in contrast to carmin, either with it or following it.	Lee, p. 212. Ehrl. I, p. 631.
Cochineal, p. 92			For general discussion, see Ehrl. I, p. 238–42.
Alone, aqueous.		Only for objects containing iron, aluminum, etc.	
Alone, alcoholic.	Mayer's old formula	Especially for arthropod tissue.	Lee, p. 143.
Tincture, (with CaCl & AlCl.)	Mayer.	For sections and staining in bulk. Used like paracarmine (see below).	Mayer, 1878. Mayer, 1892. Lee, p. 144.
Alum-cochineal.	Mayer. Czokor. Partsch.	For staining in bulk; not suited for sections. (Stains nuclei violet red, blood and muscle cells orange; colors cytoplasm weakly.)	Mayer, 1878. Mayer, 1892. Czokor, 1880. Chamberlain, p. 53.
Chromalum cocchineal.	Hansen.	For sections.	Hansen, 1905.
Iron alum cochineal (i. e. following iron alum).	Spuler.	For staining in bulk for photographing; also for sections; brings out nuclei, blood in tissues, and muscle striations.	Spuler, 1901. Lee, p. 140.

Name of stain, mordant, etc. Also page ref.	Name or author of technic	Application	Bibliographic references
Ferri-cochineal (i. e. with iron alum.)	Hansen	For sections	Lee, p. 140
Carmin, p. 93		For staining by injection and for bulk staining. Valuable in embryology.	For general discussion, see Ehrl. I, p. 167–72.
Aceto-carmin	Schneider.	As nuclear stain for sections; for fresh chromosomes in smear preparations.	Lee, p. 138.
Ammonia carmin	Ranvier Hoyer.	For sections.	Lee, p. 140.
	Van Vijhe.	For injection.	Id.
Magnesia carmin	Mayer.	For sections, and for bulk staining.	Mayer, 1892. Mayer, 1896. Lee, p. 140.
Borax carmin (alcoholic).	Grenacher. Mayer.	A much used stain for sections.	Mayer, 1892, 1896. Lee, p. 141. Chamberlain, p. 52.
Hydrochloric carmin (alcoholic).	Mayer.	For sections, and for bulk staining.	Mayer, 1892, 1896.
Alum carmin.	Grenacher.	As nuclear stain for sections.	Grenacher, 1879. Lee, p. 136–8.

With AlCl. "Muci-carmin"	Mayer.	For mucin, in sections.	Mayer, 1892, 1896.
Iron carmin.		For sections.	Lee, p. 139.
Picro-carmin (with picric acid).		For double staining effect in sections, particularly of nervous tissue. Nuclei red; cytoplasm yellow.	Lee, p. 140–1.
Carminic acid, p. 93			For general discussion, see Ehrl. I, p. 172–5.
Carmalum (with alum).	Mayer.	As nuclear stain for sections.	Mayer, 1892, 1899. Lee, p. 137. Chamberlain, p. 53.
	Rawitz.	Prepared with glycerin; for sections.	Rawitz, 1899. Lee, p. 138.
Muci-carminic acid (acid, with AlCl.)	Rawitz.	For mucin, in sections.	Rawitz, 1899.
Para-carmin (with AlCl and $CaCl_2$).	Mayer.	For sections and staining in bulk; ordinarily stains cytoplasm as well as nuclei.	Mayer, 1892, 1899. Lee, p. 142.
With iron.	Weigert. v. Wellheim.	Following treatment with iron chloride; for sections and staining in bulk.	Lee, p. 139.
	Zacharias.	Citrate of iron, following an aceto-carmine.	Lee, p. 139.

Name of stain, mordant, etc. Also page ref.	Name or author of technic	Application	Bibliographic references
	Unna.	In alcoholic solution; for elastic tissue.	Mal. & Wr., p. 123. Ehrl. I, p. 294. Lee, p. 353.
	Israel.	Dissolved in acetic acid solution. Stains nuclei blue, cytoplasm red.	Lee, p. 212. Ehrl. II, p. 328.
Orcein, p. 94	Moll.	In HCl solution; for sections of embryos.	Lee, p. 376. Ehrl. II, p. 328.
	Unna.	For connective tissue; following polychrome methylene blue.	Lee, p. 351. Ehrl. I, p. 294.
	Unna.	For plasma fibrils of epithelium; following anilin blue.	Lee, p. 340.
Brazilin, p. 95	Mayer	With alum, as a nuclear stain (like haemalum). Called brazilum	Lee and Mayer, 1907, p. 218. Lee, p. 211 Ehrl. I, p. 158.
	Hickson.	As nuclear stain, following iron alum.	Hickson, 1901. Lee, p. 211. Ehrl. I, p. 158.
Haematoxylin and haematein, p. 96			For general discussion, see Ehrl. I p. 593–605.

Alone		As nuclear stain for plant sections.	Ehrl. I, p. 598.
		As reagent for iron and copper.	Id.
With aluminium (alum-hematoxylin).	Böhmer.	Obsolete tissue stain; historic interest only.	Böhmer, 1865.
	Delafield.	As nuclear stain for tissues. For cellulose walls of plants.	Lee, p. 151–5. Chamberlain, p. 48–50
	Ehrlich.	As nuclear stain for tissues.	Ehrl. I, p. 599–600.
Hemalum (hematein with alum).	Mayer, and later modifications.	As nuclear stain for tissues.	Mayer, 1891, 1899. Lee, p. 152. Ehrl. I, p. 595.
Hemacalcium (hematein with AlCl and $CaCl_2$)	Mayer	As nuclear stain for tissues.	Mayer, 1891, 1899. Lee, p. 155. Ehrl. I, p. 596.
Muc-hematein (with AlCl and glycerin).	Mayer.	As stain for mucin.	Mayer, 1891, 1899. Ehrl. I, p. 596.
With chromium	Heidenhain, R	An early technic for tissue staining; still used.	Chamberlain, p. 44–8.
	Apathy. Henneguy. Hansen. Schultz.	Recent modifications; for general tissue staining.	Lee, p. 156–7. Ehrl. I, p. 600.
	Weigert.	For nervous tissue.	Ehrl. II, p. 229.

Name of stain, mordant, etc. Also page ref.		Name or author of technic	Application	Bibliographic references
Haemetoxylin (*continued*)	Following an iron salt.	Benda.	An obsolete technic; the original method.	Ehrl. I, p. 601.
		Heidenhain, M.	One of the most useful present histological and cytological stains, both in botany and zoology.	Lee, p. 147–51. Ehrl. I, p. 600–1.
		Bütschli. Weigert. Hansen. Kaiser.	Modifications for various special purposes.	Ehrl. I, p. 601.
	Following copper	Benda.	For studying spermatogenesis.	Id.
		Bensley.	For chromosomes and mitochondria.	Guyer, 1917, p. 145.
	With phospho-molybdic acid.	Mallory.	For central nervous system.	Mal. & Wr., p. 71. Lee, p. 157.
	With phospho-tungstic acid.	Mallory.	A very valuable pathological stain, especially for fibri in tumor tissue.	Mal. & Wr., p. 72. Ehrl. I, p. 602. Lee, p. 158.
	With vanadium.	Heidenhain, M.	For general tissue.	Ehrl. I, p. 604. Lee, p. 157.
	Double staining.	Van Gieson.	Followed by picric acid and acid fuchsin. Sometimes followed by picric acid, or following eosin, orange G, safranin or acid fuchsin.	

TABLE 3. A LIST OF BIOLOGICAL STAINS GROUPED ACCORDING TO THE FIELD IN WHICH USED.*

ANIMAL HISTOLOGY

Nuclear stains (basic)

Janus green B.
Thionin
Methylene blue
Toluidin blue
Cresyl violet
Safranin
Magdala red
Auramin
Fuchsin
Hoffman's violet
Iodine green
Gentian violet (including crystal and methyl violets)
Cochineal and **carmin**
Orcein
Brazilin
Haematoxylin

Cytoplasm stains (acid)

Picric acid
Orange G.
Bordeaux red
Fast yellow (bone tissue)
Methyl orange (for keratin in skin)
Amaranth (nervous tissue)
Biebrich scarlet W. S.
Bismarck brown
Congo red
Trypan red (vital staining)
Benzopurpin 4B
Trypan blue (vital staining)
Alizarin red S (for nervous tissue)
Neutral red (largely for vital staining)
Nigrosin
Malachite green
Light green SF yellowish
Acid fuchsin
Methyl blue
Anilin blue W. S.
Rhodamine
Eosin Y.
Eosin, alc. sol.
Erythrosin (nervous tissue)
Indigo carmin

Fat stains

Sudan III
Sudan IV
Nile blue sulfate.

*In this table the stains in bold faced type are those of widest application. When a stain is specified for practically only one purpose that purpose is mentioned in parenthesis; the stains not so designated are of fairly general application in the particular field under which they are listed.

For lignified cell walls

Methylene green
Safranin
Iodine green
Gentian violet (including crystal and methyl violets)
Methyl green

For cellulose walls

Congo red (plant mucin)
Malachite green (for host tissue in case of fungus diseases)
Light green SF yellowish (cellulose walls)
Acid fuchsin
Eosin Y
Erythrosin.
Haematoxylin

CYTOLOGY

General nuclear stains (basic)

Thionin
Methylene blue
Toluidin blue
Magdala red
Fuchsin
Gentian violet (including crystal and methyl violets)
Methyl green
Carmin
Orcein
Haematoxylin

Special chromatin stains

Alizarin red S
Thionin
Methyl green
Safranin
Gentian violet (including crystal and methyl violets)
Iodine green
Carmin (for fresh cells)
Haematoxylin

Cytoplasm stains (acid)

Picric acid
Orange G.
Bordeaux red
Methyl orange
Acid fuchsin
Eosin Y.

Stains for mitochondria, etc.

Aurantia
Janus green B
Acid fuchsin
Crystal violet.

Nuclear stains (basic)

Thionin
Methylene blue
Toluidin blue
Safranin
Cresyl violet
Fuchsin
Hoffman's violet
Iodine green
Gentian violet (including crystal and methyl violets)
Pyronin
Cochineal and **carmin**
Orcein
Haematoxylin

Cytoplasm stains (acid)

Picric acid
Martius yellow (for cancer tissue)
Orange G.
Methyl orange
Amaranth (for nervous tissue)
Biebrich scarlet W. S.
Congo red
Alizarin red S.
Neutral red
Nigrosin
Malachite green
Light green SF yellowish
Acid fuchsin
Methyl blue
Anilin blue, W. S.
Eosin Y.
Erythrosin

Blood stain constituents

Orange G. (acid)
Narcein (acid)
Methylene blue (basic)
Methylene azure (basic)
Neutral red (acid)
Acid fuchsin (acid)
Methyl green (basic)
Pyronin (basic)
Rhodamine (acid)
Eosin Y (acid)

Fat stains

Sudan IV
Nile blue sulfate.

Bacterial stains

Bismarck brown (Gram counterstain)
Thionin
Methylene blue
Safranin (Gram counterstain)
Fuchsin
Gentian violet (including crystal and methyl violets)
Methyl green (constituent of Pappenheim stain)
Pyronin
Erythrosin
Rose bengal

Used in bacteriological media

Neutral red
Fuchsin
Acid fuchsin
Brilliant green
Methylene blue
Eosin Y.

APPENDIX II

COMMISSION SPECIFICATIONS OF CERTAIN STAINS

As announced in several notes published by the Commission in Science, it is planned to draw up specifications for all the stains put on a certification basis. So far as these specifications have been prepared they are given on the following pages. Similar specifications for other stains will be prepared later.

It must be understood that these specifications are not intended to furnish definite statements as to the chemistry of satisfactory stains. The object with which they were drawn up was to allow the manufacturers as much latitude in the matter of chemical composition as has been found consistent with good results in practice, and to lay the greatest stress upon the performance of the stains in actual laboratory use. The requirements listed are those which must be met by dyes submitted to the Commission for certification.

These specifications are published, moreover, with the understanding that they are subject to revision at any time. Further investigations are in progress concerning the adaptability of various dye products for different purposes, and also as to the relation of optical characters to performance in staining. The Commission reserves the right to modify the specifications for any stain in regard to either of these two points as data accumulate to show the need of modification.

SPECIFICATIONS FOR METHYLENE BLUE

1. Samples of methylene blue to be considered must be of the so-called medicinal grade. It is expected that they will meet the U. S. P. requirements, but less weight will be attached to this consideration than to those following. In other words, a sample giving satisfactory performance will not be excluded because of failure in some particular to meet these chemical requirements.

2. Methylene blue for the purpose above specified must contain at least 75 per cent total color, this to be determined by reduction with titanous chloride. When reduced by titanous chloride in an atmosphere of carbon dioxide, 1 gram of the dye must consume at least 4.69 cc. normal titanous chloride solution.

3. The methylene blue must have no solvent action on casein. This is to be determined as follows: Prepare two 1 per cent solutions of this stain, one in distilled water, the other in tap water. Place single drops of skimmed milk on each of two clean glass slides and smear each drop over a surface of about one square centimeter so as to form a very thin film of milk; allow this film to dry without heat or at a temperature not over 60°C., immerse for about a minute in xyol to dissolve the fat, then for the same length of time in alcohol to coagulate the casein. After this immerse one slide in the distilled water solution of methylene blue and the other slide in the tap water solution, allowing them to stand for three minutes; at the end of this period there should be no action of the stain on the casein.

4. The methylene blue should stain the diphtheria organism in any of the types of solutions ordinarily employed. It should be tested as follows: Prepare three solutions of the stain, one a 1 per cent solution in distilled water, the second a mixture of three parts saturated alcoholic solution to 10 parts of distilled water,

and the third three parts of saturated alcoholic solution to 10 parts of 0.01 per cent NaOH. Prepare three slides of a fresh culture of a diphtheria organism; stain one slide in each of these three solutions for two or three seconds only, i. e., just as briefly as the stain can be poured on and poured off, and wash each slide immediatly. Examined under the microscope all three of these preparations should show deeply stained bacteria with the characteristic metachromatic granules sufficiently distinct to insure accurate diagnosis.

5. The sample should prove satisfactory for histological use. No exact method for determining this can be given, but the sample must be submitted to one or two experts in histological technic in order to get their judgment.

6. It must be understood that these standards refer to samples to be used for ordinary bacteriological and histological staining. Special standards for methylene blue used in vital staining will undoubtedly be necessary. These standards, however, have not yet been determined.

SPECIFICATIONS FOR SAFRANIN O

1. Samples of safranin O must be of the type represented by Colour Index No. 841 and on spectrometric analysis should have an absorption curve maximum at approximately 515$\mu\mu$ as determined in a one cm. layer by a spectrophotometer. Other dyes must not be present.

2. Safranin samples to be certified by the Commission must contain at least 75 per cent total color as determined when reduced by titanous chloride in an atmosphere of carbon dioxide. One gram of the dye must consume at least 4.195 cc. normal titanous chloride solution.

3. The samples should prove satisfactory for histological use. No exact method for determining this can be given, but the samples must be submitted to one or two experts in histological technic in order to get their judgment. Their judgment must be based to a considerable extent upon the behavior of the stain in the Flemming triple staining technic, in which it is used together with orange G and gentian violet. In other words, the stain must be of such a shade as to contrast well with both of these two other dyes.

SPECIFICATIONS FOR BASIC FUCHSIN

1. Basic fuchsin designed for staining and indicator purposes must be rosanilin or new fuchsin (Colour Index No. 678) or else a mixture of rosanilin and pararosanilin containing at least half of the former (that is, corresponding to Colour Index No. 677).

2. Fuchsin samples to be certified by the Commission must be of such a strength that, when reduced by titanous chloride in an atmosphere of carbon dioxide, one gram of the dye will consume at least 46.5 cc. normal titanous chloride solution. A sample of this strength will be between 76 and 85 per cent total dye content, the exact dye content varying according to the relative amounts of the higher and the lower homologs present.

3. The sample should prove satisfactory for staining the tubercle organism and should retain its color sufficiently when treated by the Ziehl method to be diagnostic when staining tubercular discharges. This must be determined by an investigator skilled in this particular technic.

4. The sample must prove satisfactory for use in the Endo medium. In making this test the following technic should be used: A saturated alcoholic solution is diluted 2 to 3 times, the dilution to be such that no precipitation occurs when mixed with a sodium sulphite solution. Then add 0.5 cc. of the saturated and the diluted fuchsin solutions each to 10 cc. of a 2.5 per cent sodium sulphite solution. Select the strongest of these which shows no precipitate and add it to the other ingredients of Endo agar, sterilize and cool. It should then be colorless, but the color must be restored by the colon and dysentery organisms when inoculated upon it. The test must be made by one familiar with the technic in question.

5. It must be understood that as basic fuchsin is used in other special forms of technic, new standards may be called for. The present specifications apply particularly to the above mentioned two uses: but samples fulfilling them are ordinarily satisfactory for all histological purposes.

SPECIFICATIONS FOR ACID FUCHSIN

1. Acid fuchsin designed for staining and indicator purposes must correspond to Colour Index No. 692. Inasmuch as the dyes of this type on the market are mixtures of greatly varying composition, and it has not yet been shown that any one of the components of these mixtures is better for biological purposes than any of the others, no definite physico-chemical specifications are made at the present time, with the reservation that more specific requirements may be drawn up at a later date if future work shows them to be desirable.

2. Acid fuchsin samples to be certified by the Commission should be of such a strength as to consume not less than 2.0 cubic centimeters of normal $TiCl_3$ solution per gram of sample. Such samples would have a total dye content of approximately 60 per cent.

3. Acid fuchsin samples for certification should prove satisfactory counterstains in general histological work. They should in particular prove satisfactory in the Bensley technic for staining mitochondria, and in the Mallory connective tissue stain (with anilin blue and orange G). They should be tested for these purposes by histologists skilled in the technic in question.

4. The samples shall prove satisfactory for use in the Andrade indicator. For this purpose they shall be tested as follows: Dissolve 0.2 gram in 100 cc. water, neutralize by adding between 15 and 25 cc. of Normal NaOH. When neutralized with the proper amount of the reagent, the solution should become a straw color upon standing and should impart no noticeable color to ordinary bacteriological nutrient agar when added at the rate of 1 per cent. The red color should be restored in this medium by the colon organism when growing in the presence of any sugar attacked by it.

5. Labels to be used on the acid fuchsin samples should state the formula giving best results with the sample in question in preparing the Andrade indicator.

SPECIFICATIONS FOR GENTIAN AND CRYSTAL VIOLET

1. Crystal violet samples shall contain no dye except hexa-methyl pararosanilin (Colour Index No. 681), having its absorption maximum at 590$\mu\mu$ when determined in a cell of 1 milimeter thickness in a spectrophotometer.

2. Crystal violet samples to be certified by the Commission must be of at least 80 per cent dye content as determined by reduction of $TiCl_3$ in an atmosphere of carbon dioxide.

3. Gentian violet for general staining purposes, as defined by the Commission on the Standardization of Biological Stains, must be either penta-methyl or hexa-methyl pararosanilin, or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B. In other words the dye must be Colour Index No. 680 or No. 681.

4. Gentian violet samples to be certified by the Commission must be of at least 75 per cent dye content as determined by reduction of $TiCl_3$ in an atmosphere of carbon dioxide. The diluent used must be dextrin.

5. The sample of crystal or gentian violet should prove satisfactory for staining bacteria according to the Gram technic when made up in a 0.5 per cent aqueous solution, without the use of anilin oil or any other mordant, and stained by the following procedure: crystal or gentian violet 1 minute, Lugol's iodine solution 1 minute, 95 per cent alcohol 30 seconds, 0.05 per cent safranin solution 10 seconds. In making the test, a weakly Gram-positive orgnism and a Gram-negative organism should be tested.

6. The sample should prove satisfactory in the Flemming triple stain when used with safranin and orange G. For this purpose it must be tested by histologists skilled in the technic in question. It should also prove a satisfactory nuclear stain in general histology.

7. Either gentian violet or crystal violet for certification must have sufficient bacterostatic power so that, when added to nutrient agar in the proportion of one part to a million, it will entirely prevent the growth of *Bacillus subtilis* when this organism is streaked over the surface of the hardened agar. It must be understood, however, that this bacterostatic property of the dye is still under investigation and has a relation not yet fully understood to the control of certain diseases. As new investigations bring new light on the subject it may be necessary to draw up new specifications to cover this point.

SPECIFICATIONS FOR PYRONIN

1. Pyronin designed for staining purposes must be either pyronin G (Colour Index No. 739) or pyronin B (Colour Index No. 741). The container in which it is sold should be marked plainly as to which of these two dyes is furnished.

2. Samples of pyronin G must be characterized by an absorption curve showing a maximum absorption at about $545\mu\mu$ when determined in a 0.002 per cent solution in a layer 1 milimeter thick. To be certified by the Commission these samples should be of such a strength that the extinction coefficient at the point of maximum absorption shall be not less than 1.20.

3. Samples of pyronin B tested in the spectrophotometer under the above mentioned conditions must have an absorption maximum at about $550\mu\mu$, and must be of such a strength that the extinction coefficient at the point of maximum absorption shall be not less than 1.00.

4. The samples shall be satisfactory for use in the Pappenheim combination with methyl green. In the case poor results are obtained with the formulae for

this stain commonly found in the literature it should be suspected that the trouble might be due to the greater concentration of pyronins now available and the tests should be made by the following formula calling for a smaller proportion of pyronin:

Methyl green	100 gm.
Pyronin	25 gm.
Alcohol	5. cc.
Glycerin	20 cc.
2 per cent carbolized water	100 cc.

SPECIFICATIONS FOR EOSIN Y

1. Samples of eosin Y must be of the type represented by Colour Index No. 768. They must, furthermore, be the dibasic sodium salt of tetra-brom fluorescein having an absorption maximum at approximately 516$\mu\mu$ as determined in a .002 per cent solution in a layer 1 cc. thick in a spectrophotometer. They should be readily soluble in water and alcohol (insoluble material not over 0.5 per cent).

2. Eosin samples to be certified by the Commission must contain at least 85 per cent total color as determined when reduced by titanous chloride in an atmosphere of carbon dioxide.

3. Eosin samples must yield a satisfactory Wright's stain in combination with methylene blue by the formula given in Mallory and Wright (1924) page 470. The quality of the resulting compound dye must be judged by someone skilled in its use for staining blood.

4. The samples must prove satisfactory for counterstaining against basic dyes in histological technic, their performance to be judged by experts in the staining procedures involved.

5. Eosin should prove satisfactory with methylene blue in the Levine eosin-methylene-blue medium for the differentiation of certain organisms of the colon typhoid group. In making this test the method should be followed which is published by Levine (1921) p. 62–4.

SPECIFICATIONS FOR ORANGE G

1. Samples of orange G submitted for certification must be of the type listed in the Colour Index as No. 27 and must be characterized by an absorption maximum at approximately 485$\mu\mu$ as determined in a .004 per cent solution in a layer 1 cc. thick in a spectrophotometer.

2. The total dye content of the samples must be at least 80 per cent as determined by reduction of titanous chloride in an atmosphere of carbon dioxide.

3. The samples must prove satisfactory for counterstaining in histology and cytology, giving a clear orange shade and in a brown tone to the cytoplasmic bodies. They shall also be satisfactory for the Flemming triple stain, in which this dye is used in contrast to safranin and crystal violet. Its performance in these procedures shall be judged only by ones skilled in the technic in question.

SPECIFICATIONS FOR HAEMATOXYLIN

1. A sample of haematoxylin to be considered for certification must consist of well defined crystals showing sandy or light brown color and shall contain less than 0.1 per cent ash. With NaOH it shall yield a purple solution turning brown on

standing in presence of the air; with lead acetate it shall yield first a colorless and then a blue precipitate darkened by the air.

2. When used by one of the standard methods such as that of Haidenhain for staining actively growing root tips or other good cytological material showing mitosis, it shall give a sharp, vigorous black staining of the chromosomes. When used in the Delafield method, it must give a clear-cut blue picture of chromatic material. These tests shall be made by someone skilled in the technic involved.

3. Solutions shall show no discoloration and shall retain their staining qualities upon standing for a period of three or four weeks.

SPECIFICATIONS FOR THIONIN

1. Samples of thionin (Syn. Lauth's violet) must correspond to Colour Index No. 920 and be characterized by an absorption curve with a maximum at about 602$\mu\mu$ as determined in a .001 per cent solution in a layer 1 cc. thick in a spectrophotometer.

2. Samples submitted for certification must have a dye content of at least 85 per cent as determined by titanous chloride reduction in an atmosphere of carbon dioxide.

3. The samples should prove satisfactory for staining frozen sections of fresh animal tissue and should show good metachromatic effects when applied in a 1 per cent solution for 1 to 5 minutes, followed by rapid washing and mounting in water. Their performance in this matter shall be judged by someone familiar with the technic.

4. The samples should prove satisfactory for staining bacteria by the "little plate" technic described by W. D. Frost in Jr. Inf. Dis. **19,** (1916) p. 273–287

APPENDIX III
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